MODEL-BASED EVALUATION OF ENZYMATIC HYDROLYSIS OF MICROALGAL CELLULOSE AND DIFFERENT CELLULOSIC MATERIALS

HANIEH SHOKRKAR

Biotechnology Research Center, Faculty of Chemical Engineering,
Sahand University of Technology, Tabriz, Iran
✉ *Corresponding author: h_shokrkar@sut.ac.ir*

Received November 22, 2022

The development of a kinetic model for fermentable sugar production is a significant issue due to the complexity of the enzymatic hydrolysis of cellulose. This study presents a proper mathematical model for the evaluation of enzymatic hydrolysis of microalgal cellulose and different cellulosic materials. The modeling results were compared with experimental results of enzymatic hydrolysis of microalgal cellulose and different cellulosic materials. Also, the results of the proposed modified model and another model from the literature were compared. The comparison indicated that the proposed modified model gives a more accurate prediction of the production of glucose, cellobiose, and cellulose consumption as a function of time, during enzymatic hydrolysis of cellulosic materials. The proposed modified model, with an average of $\chi^{2(m)}$ equal to 38.15, is more accurate than the previously reported model, with an average of $\chi^{2(m)}$ equal to 48.84.

Keywords: cellulosic material, enzymatic hydrolysis, kinetic, modified model, microalgae

INTRODUCTION

Industrialization and the fast growth rate of the world population have increased the consumption for energy.[1] The use of fossil fuels as an energy source has serious environmental implications, such as global warming and air pollution, among others. Therefore, providing energy from renewable green sources is crucial.[2] The first-generation biofuels are not feasible options for biofuel production as the human demand for food has yet to be met. Also, the limitations of second-generation biofuels, such as complex techniques required for pretreatment processes, has led to the third-generation biofuels – those based on algae.[3] There are many efforts to decline carbon dioxide emissions and expand other energy sources as alternatives to fossil fuels.[4] Algae would be qualified candidates for green energy sources, procuring energy from sunlight and making their biomass by removing carbon dioxide from the atmosphere through photosynthesis.[5] Considering their fast growth and low-cost downstream processes, microalgae are considered important sources of biofuel.[6] Microalgal carbohydrates are commonly cellulose, so microalgae are more quickly hydrolyzed to fermentable sugars than other cellulosic materials.

Cellulose is a principal constituent of all plant materials and a linear biopolymer of glucose.[7,8] Enzymatic hydrolysis of cellulose is performed by cellulase enzymes, including endo-glucanase, exo-glucanase (cellobiohydrolases), and β-glucosidase (cellobiase).[9,10] Enzymatic hydrolysis has various advantages compared to acid hydrolysis, including lower equipment wear, fewer corrosion problems, higher glucose yield, without sugar degradation and inhibitory product production.[11-13] However, enzymatic hydrolysis of cellulose is still considered the main bottleneck in the production of bioethanol due to the low hydrolysis rates and the high cost of enzymes.[14-16] Many parameters, including cellulose structure (accessible area, degree of polymerization, crystallinity) and cellulase system (enzyme activities, adsorption, synergism, and inhibition) have an essential role in the enzymatic hydrolysis of cellulose.[17] However, because of the difficulty in the measurement of parameters, such as crystallinity and degree of polymerization of cellulose, a model based on observable properties

would help to analyze fermentable sugars or bioethanol production.

Several authors investigated the enzymatic hydrolysis kinetics of cellulosic material by applying Michaelis–Menten's (M-M) equation due to its simplicity.[18-23] Also, Ye and Berson[24] proposed a mathematical model describing the kinetics of glucose formation from cellulose with first-order inactivation of adsorbed cellulase. In this model, substrate reactivity (transformation in the degree of polymerization, crystal structure, substrate availability, *etc.*), conversion of cellulose to cellobiose, and cellobiose to glucose have not been included. Some authors believe that substrate reactivity is an influencing parameter on the enzymatic hydrolysis of cellulosic materials.[25-30] They detected cellulose hydrolysis rate increased 3–30 times in amorphous cellulose, compared to crystalline cellulose.

The objective of this work was to modify the model proposed by Ye and Berson,[24] to take into consideration substrate reactivity, enzyme inactivation, and the kinetics of cellobiose and glucose production. Afterwards, the modified model and another model suggested by Zheng *et al.*[22] were compared for the prediction of enzymatic hydrolysis results for microalgal cellulose and different cellulosic materials. The prediction accuracy was compared to actual experimental results obtained for the mentioned cellulosic materials in previous studies.

EXPERIMENTAL

Model description

In this work, the model proposed by Zheng *et al.*[22] was evaluated, using experimental results from enzymatic hydrolysis of microalgae (our previous study) and the experimental data reported in other four studies reported in the literature. Then, the modified kinetic model was used to predict concentrations of cellulose, cellobiose, and glucose during enzymatic hydrolysis. A reaction scheme for the modeling of cellulose hydrolysis is shown in Figure 1. AQUASIM software was used as a tool to simulate the enzymatic hydrolysis of cellulose.[31]

Kinetic model from the literature

The model proposed by Zheng *et al.*[22] (further referred to as Model 1) assumes that the hydrolysis of cellulose occurs in three steps. The cellulose is hydrolyzed to soluble cellobiose by the synergistic action of endo-β-1,4-glucanase (EG) and exo-β-1,4-cellobiohydrolase (CBH). The cellulose is hydrolyzed to glucose by the synergistic action of CBH and exo-β-1,4-glucanase. Cellobiose is hydrolyzed to glucose by the action of β-glucosidase. The reaction scheme (Fig. 1(a)) involves hydrolysis reactions r_1, r_2, and r_3. The reaction rates are given by the following equations:

$$r_1 = \frac{k_{1r}.E.SR.C}{1+\frac{G_2}{k_{1IG2}}+\frac{G}{k_{1IG}}} \quad (1)$$

$$r_2 = \frac{k_{2r}.E.SR.C}{1+\frac{G_2}{k_{2IG2}}+\frac{G}{k_{2IG}}} \quad (2)$$

$$r_3 = \frac{k_{3r}.E.G_2}{k_{3M}.(1+\frac{G}{k_{3IG}})+G_2} \quad (3)$$

Substrate reactivity (SR) is expressed according to the following equation:

$$SR = \frac{C}{C_0} \quad (4)$$

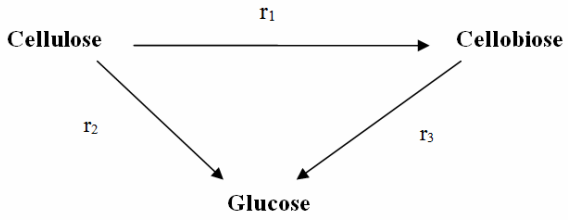

Figure 1: Reaction scheme for modeling cellulose hydrolysis according to (a) literature model; (b) modified model

This model was developed based on end-product inhibition. In these rate equations, k_{ir} (1, 2) is the reaction rate constant (mL/(mg. h)); k_{3r} is the reaction rate constant (h^{-1}); k_{1IG}, k_{1IG2} (1, 2) are inhibition constants (mg/mL) of glucose and cellobiose on enzymes, respectively; k_{3M} is cellobiose saturation

constant (mg/mL); and C, G_2, G are concentrations of cellulose, cellobiose, and glucose (mg/mL), respectively. C_0 and E are the initial substrate concentration (g/L) and enzyme concentration (mg/mL), respectively. The mass balance equations of cellulose, cellobiose, and glucose can be written as follows:

$$\frac{dC}{dt} = -r_1 - r_2 \quad (5)$$

$$\frac{dG_2}{dt} = 1.056r_1 - r_3 \quad (6)$$

$$\frac{dG}{dt} = 1.116r_2 + 1.053r_3 \quad (7)$$

Kinetic model proposed in this study

The model initiated by Ye and Berson[24] simulates the direct conversion of cellulose to glucose. However, this model does not consider substrate reactivity. Therefore, in the present study, the modification of this model was performed to consider the kinetics of cellobiose and glucose formation, substrate reactivity, and enzyme inactivation (further referred to as Model 2).

The cellulase inactivation is described in the modified model using an exponential decay term. In the modified model, it is assumed that the hydrolysis of cellulose occurs in two steps. First, cellulose is hydrolyzed to soluble cellobiose by the synergistic action of β-1,4-glucan cellobiohydrolase and endo-β-1,4-glucanase. Second, the cellobiose is hydrolyzed to glucose by the action of β-glucosidase (Fig. 1(b)). To simplify the model, r_2 in the previous model is ignored. The reaction rates and mass balance equations in the modified model are demonstrated by the following equations:

$$K_c = \frac{k_r}{k_r + k_f} + \frac{k_f}{k_r + k_f}.\exp(-(k_r + k_f).t) \quad (8)$$

$$r_1 = \frac{k_{1r}.E.SR.K_c.C}{C + K_m} \quad (9)$$

$$r_2 = \frac{k_{2r}.E.K_c.G_2}{G_2 + K_m} \quad (10)$$

$$\frac{dC}{dt} = -r_1 \quad (11)$$

$$\frac{dG_2}{dt} = 1.056r_1 - r_2 \quad (12)$$

$$\frac{dG}{dt} = 1.053r_2 \quad (13)$$

In these rate equations, k_{1r} is the reaction rate constant (mL/(mg.h)); and k_{2r} is the reaction rate constant (h^{-1}); k_m is the saturation constant (mg/mL); and C, G_2, G are concentrations of cellulose, cellobiose, and glucose (mg/mL), respectively. In addition, k_f and k_r are inactivation and reactivation rate constants (h^{-1}), respectively. SR is substrate reactivity and is expressed as described in Equation (4).

Evaluation of model predictions

The model parameters were estimated using the AQUASIM software by minimizing the sum of the squares of the weighted deviations ($\chi 2(m)$) between the experimental data and the model results. $\chi 2(m)$ is expressed as:

$$\chi 2(m) = \sum_{i=1}^{n} \left(\frac{X_{\exp,i} - X_i(m)}{\sigma_{\exp,i}}\right)^2 \quad (14)$$

In this equation, $X_{exp,i}$ is the i-th measurement, $X_i(m)$ is the calculated value of the model variable corresponding to the i-th measurement, $\sigma_{exp,i}$ is standard deviation, m is the model parameter and n is the number of data points. AQUASIM minimizes the sum of squares with the restriction $m_{min,i} << m << m_{max,i}$, where $m_{min,i}$ and $m_{max,i}$ are the minimum and maximum of the constant variable m. The numerical values of the parameters were obtained by fitting the model to experimental data taken from the literature.

Enzymatic hydrolysis of microalgal cellulose

Mixed microalgae (collected from freshwater in East Azarbayjan, Iran) have been cultured in a photo-bioreactor and then harvested, as reported in our previous study.[32] The harvested algae were oven dried at 70 °C for two days. Afterwards, the dried algae were ground into powder in a planetary ball mill and used for enzymatic hydrolysis of microalgal cellulose by cellulase enzyme. Cellulase from *Trichoderma reesei* was used for enzymatic hydrolysis experiments.

For the enzymatic hydrolysis of microalgal cellulose, the algal biomass powder was mixed with citrate buffer with concentrations of 50, 75, and 100 g/L. These samples were autoclaved at 121 °C for 15 min and then mixed with a cellulase concentration of 0.416 mg protein/mL. The temperature and pH of the enzymatic hydrolysis medium were adjusted to 50 °C and 5, respectively. Then, the samples were incubated in a shaker at 150 rpm for 72 h. Analysis samples were taken at different times during enzymatic hydrolysis, and glucose and cellobiose concentrations were measured. All experiments were operated in duplicate.

Glucose and cellobiose concentrations were measured using high-performance liquid chromatography. The determination of the total carbohydrate content of microalgae was performed using the Anthrone method.[33] The glucose yield can be calculated according to the following equation:

$$\text{Glucose yield\%} = \frac{\text{Extracted glucose (g)}}{\text{Total carbohydrate in algae (g)}} \times 100 \quad (15)$$

RESULTS AND DISCUSSION

The present study aimed at developing a suitable mathematical model for fermentable sugar production from various cellulosic raw materials. Thus, the model proposed by Ye and Berson[24] was modified in order to take into account the complexity of the enzymatic hydrolysis of cellulose, and namely, aspects such as substrate reactivity, enzyme inactivation, and the kinetics of cellobiose and glucose production. This modified version of the model was evaluated in comparison with another established model proposed by Zheng *et al*.[22] The evaluation was performed based on the conditions of enzymatic hydrolysis applied in previous studies: namely, the enzymatic hydrolysis of microalgal cellulose (our previous study),[32] and the hydrolysis of various other cellulosic materials: insoluble cellulose (Fan and Lee[34]), cotton stalk (Gusakov *et al*.[35]), non-crystalline cellulose (Peri *et al*.[19]) and creeping wild ryegrass (Zheng *et al*.[22]). Therefore, the experimental results obtained in our previous study and others reported in the literature were evaluated by solving differential equations using AQUASIM software. The model parameters were estimated by minimizing the sum of the squares of the weighted deviations between measurements and calculated model results. These will be further discussed below.

In our previous study,[32] at the end of microalgae cultivation in a photo-bioreactor, the algal biomass concentration (TSS) was measured to be 2.05 g/L. Also, the microalgae had a total carbohydrate content of about 21% of TSS. As previously mentioned, enzymatic hydrolysis of microalgal cellulose was performed with initial microalgae concentrations of 50, 75, and 100 g/L, cellulase concentration of 0.416 mg protein/mL at 50 °C, and pH 5 for 72 h. The main products resulting from the enzymatic hydrolysis of the cellulosic material by cellulases are glucose and cellobiose.

Microalgae contain carbohydrates, such as cellulose, often accumulating in the cell wall. Therefore, cell wall disruption is necessary for using the carbon source during the fermentation process.

Table 1
Estimate values of parameters derived from the kinetic model from the literature by Zheng *et al*.[22] (Model 1)

Parameters	Estimate values (our study on microalgae)[32]	Estimate values based on[34]	Estimate values based on[35]	Estimate values based on[19]	Estimate values based on[22]
k_{1IG2} (mg/mL)	0.04	0.04	0.04	0.04	0.04
k_{1IG} (mg/mL)	0.10	0.10	0.10	0.10	0.10
k_{1r} (mL/(mg.h))	14.70	7.62	0.294	160.2	16.5
k_{2IG2} (mg/mL)	132.50	132.50	132.50	132.50	132.50
k_{2IG} (mg/mL)	0.01	0.01	0.01	0.01	0.01
k_{2r} (mL/(mg h))	13.23	1.248	0.168	99.60	7.08
k_{3M} (mg/mL)	25.50	25.50	25.50	25.50	25.50
k_{3IG} (mg/mL)	2.10	2.10	2.10	2.10	2.10
k_{3r} (h^{-1})	10.06	1.626	0.204	312.60	267.60
$\chi 2(m)$	10.12	80.52	57.810	11.65	84.11

Table 2
Estimate values of parameters derived from the modified version of the model of Ye and Berson[24] (Model 2)

Parameters	Estimate values (our study on microalgae)[32]	Estimate values based on[34]	Estimate values based on[35]	Estimate values based on[19]	Estimate values based on[22]
k_r (h^{-1})	0.033	0.033	0.033	0.033	0.033
k_f (h^{-1})	0.251	0.251	0.251	0.251	0.251
k_{1r} (mL/(mg. h))	41.48	10.86	0.36	612	42.30
K_m (mg/mL)	38.638	38.638	38.638	38.638	38.638
k_{2r} (h^{-1})	46.26	7.02	0.36	1320.60	852.60
$\chi 2(m)$	11.03	59.39	28.42	5.49	59.16

To determine the kinetic parameters, the effects of microalgae biomass concentrations of 50, 75, and 100 g/L on enzymatic hydrolysis of microalgal cellulose were investigated with constant cellulase concentration of 0.416 mg protein/mL at 50 °C, and pH 5 for 72 h.

The comparison between measured and predicted concentrations of cellobiose, glucose, and glucose *versus* time is demonstrated in Figure 2. Also, the estimated parameters from the two models are shown in Tables 1 and 2.

Figure 2 shows that the increment in the microalgal biomass concentration from 50 to 100 g/L leads to a reduction in the initial rate of enzymatic hydrolysis. Also, Figure 2 shows that the highest glucose yield was 56% after 72 h, when the microalgal biomass concentration of 50 g/L was applied. When the microalgal biomass concentration was increased from 50 to 100 g/L, the glucose yield declined from 56% to 45%.

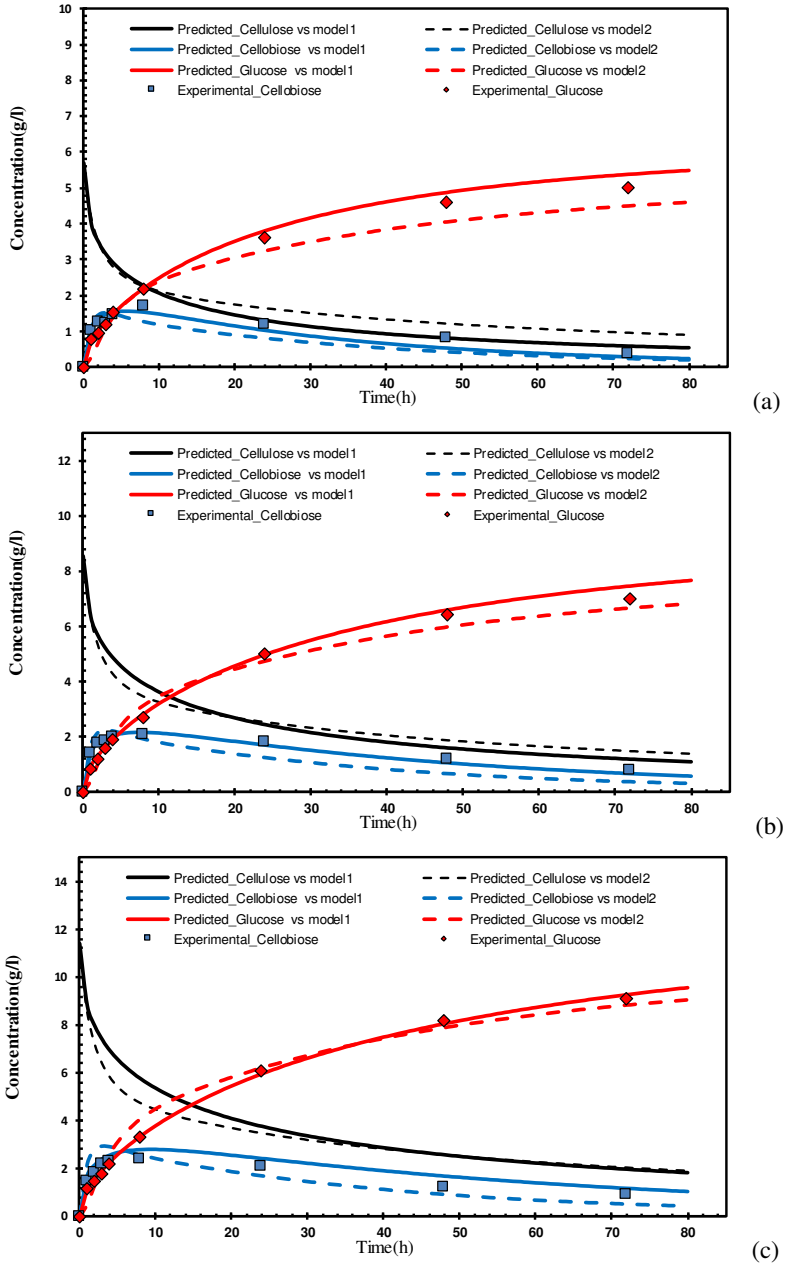

Figure 2: Comparison between predicted and experimental results for glucose, cellobiose and cellulose during enzymatic hydrolysis of cellulose with initial microalgal concentrations of (a) 50 g/L; (b) 75 g/L; (c) 100 g/L

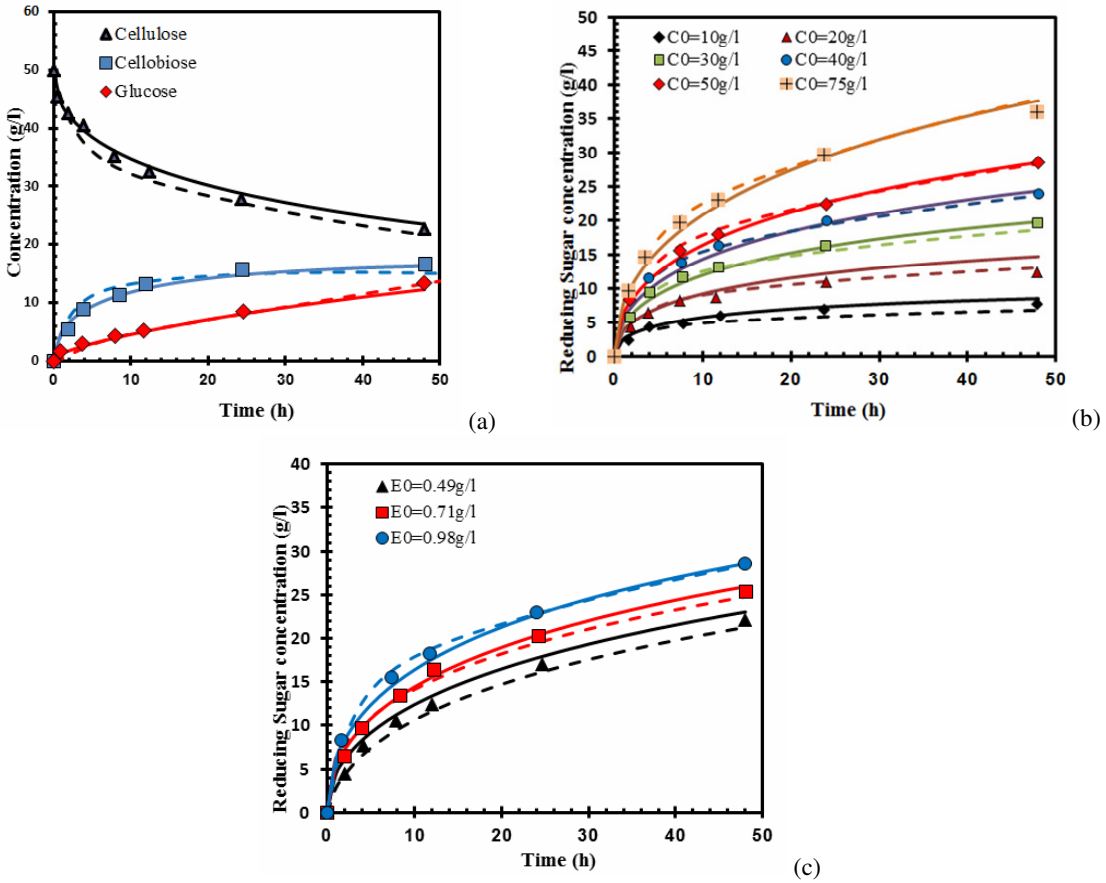

Figure 3: Comparison between predicted and experimental results for (a) glucose, cellobiose and cellulose during enzymatic hydrolysis of cellulose at initial substrate concentration of 50 g/L, and cellulase concentration of 0.98 g/L; (b) reducing sugar concentration at different initial cellulose concentration (10, 20, 30, 40, 50, 75 g/L) and cellulase concentration of 0.98 g/L; (c) reducing sugar concentration at different initial enzyme concentration (0.49, 0.71, 0.98 g/L) and initial substrate concentration of 50 g/L (solid and dashed lines show the simulation results derived from Models 1 and 2 respectively)

Thus, it can be concluded that biomass concentration is an important factor, which can be optimized to gain the highest sugar yield. Therefore, the appropriate microalgal biomass concentration was taken as 50 g/L in the present study. As shown in Figure 2, the predicted results for Models 1 and 2 are in good concordance with the experimental results of enzymatic hydrolysis of microalgal cellulose. The sum of the squares of the weighted deviations (χ^2) has been calculated to be 10.12, and 11.03 for Models 1 and 2, respectively. Afterward, the modified model and the model from the literature by Zheng *et al.*[22] were compared to see their accuracy in predicting the experimental results reported by Fan and Lee.[34] Fan and Lee[34] performed enzymatic hydrolysis of insoluble cellulose with initial substrate concentrations of 10, 20, 30, 40, 50, and 75 g/L, and cellulase concentration of 0.98 g/L at 50 °C, pH 4.5. Also, initial substrate concentrations of 50 g/L and cellulase concentrations of 0.49, 0.71, and 0.98 g/L were investigated in their study.

Figure 3(a) compares the measured and the predicted concentrations of glucose, cellobiose, and cellulose *versus* time, with an initial substrate concentration of 50 g/L and cellulase concentration of 0.98 g/L. Also, Figure 3(b) demonstrates a comparison of the measured and the predicted reducing sugar concentration at different initial cellulose concentration (10, 20, 30, 40, 50, 75 g/L) and cellulase concentration of 0.98 g/L. The comparison between the measured and the predicted results for different initial cellulase concentrations (0.49, 0.71, 0.98 g/L) and initial substrate concentration of 50 g/L is shown

in Figure 3(c).[34] Tables 1 and 2 list best-fit estimates of kinetic parameters.

As can be seen, reducing sugar concentration depends on hydrolysis time, initial cellulose concentration, and cellulase concentration. The sum of the squares of the weighted deviation ($\chi 2(m)$) has been calculated to be 80.52 and 59.39 from Models 1 and 2, respectively. As illustrated in Figure 3(c), the experimental and predicted results indicate an increase in the reduced sugar production rates with increasing enzyme concentration. However, the hydrolysis rate did not increase at the high enzyme concentration because of the enzyme saturation on the available surface of cellulose.

Subsequently, the modified model in this study and the model proposed by Zheng *et al.*[22] were compared to predict the experimental results obtained by Gusakov *et al.*[35] Enzymatic hydrolysis of pretreated cotton stalks was performed under the following conditions: initial substrate concentration of 40 g/L and cellulase concentration of 10 g/L; initial substrate concentration of 40 g/L and cellulase concentration of 25 g/L; initial substrate concentration of 80 g/L and cellulase concentration of 40 g/L; at 50 °C, pH 4.5.[35] The comparison between the predicted and the experimental results of enzymatic hydrolysis of cellulose under conditions based on the study of Gusakov *et al.*[35] is illustrated in Figure 4. Also, the estimated parameters from the two models are shown in Tables 1 and 2.

The sum of the squares of the weighted deviations has been computed to be 57.810 and 28.42 for Models 1 and 2, respectively. The simulation results indicate that both models could successfully predict the time course production of glucose, cellobiose, and cellulose during enzymatic hydrolysis.

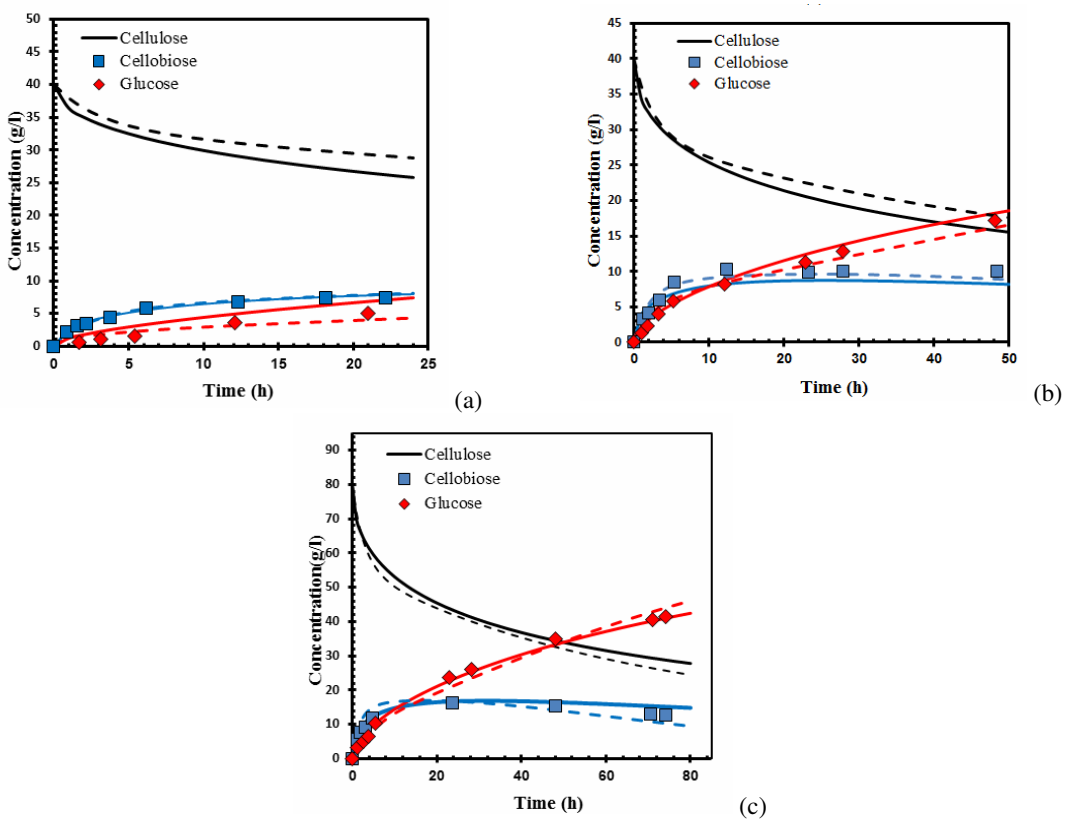

Figure 4: Comparison between predicted and experimental results for glucose, cellobiose and cellulose during enzymatic hydrolysis of cellulose at (a) initial substrate concentration of 40 g/L and concentration of cellulase of 10 g/L; (b) initial substrate concentration of 40 g/L and concentration of cellulase of 25 g/L; (c) initial substrate concentration of 80 g/L and concentration of cellulase of 40 g/L; at 50 °C and pH 4.5 (solid and dashed lines show the simulation results derived from Models 1 and 2, respectively)

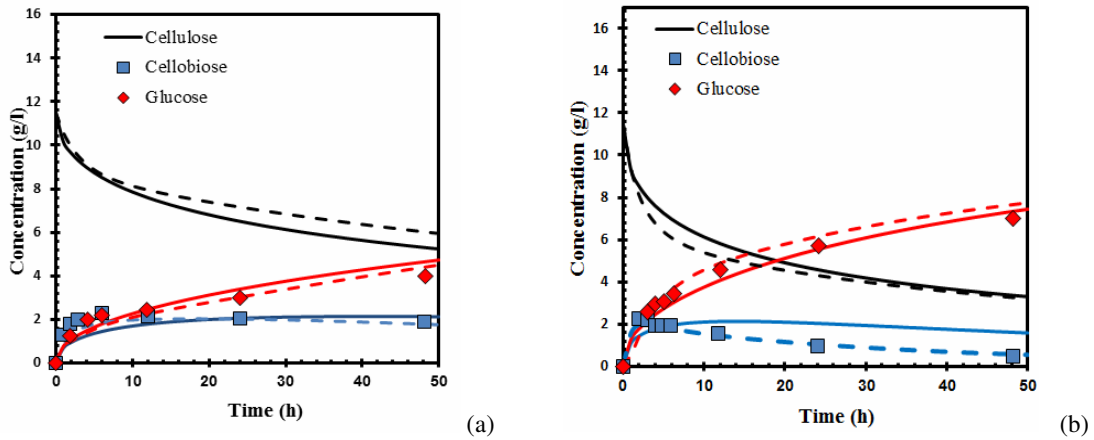

Figure 5: Comparison between predicted and experimental results for glucose, cellobiose and cellulose during enzymatic hydrolysis of cellulose at (a) 1 FPU/g-glucan enzyme loading; (b), 3 FPU/g-glucan enzyme loading (solid and dashed lines show the simulation results derived from Models 1 and 2 respectively)

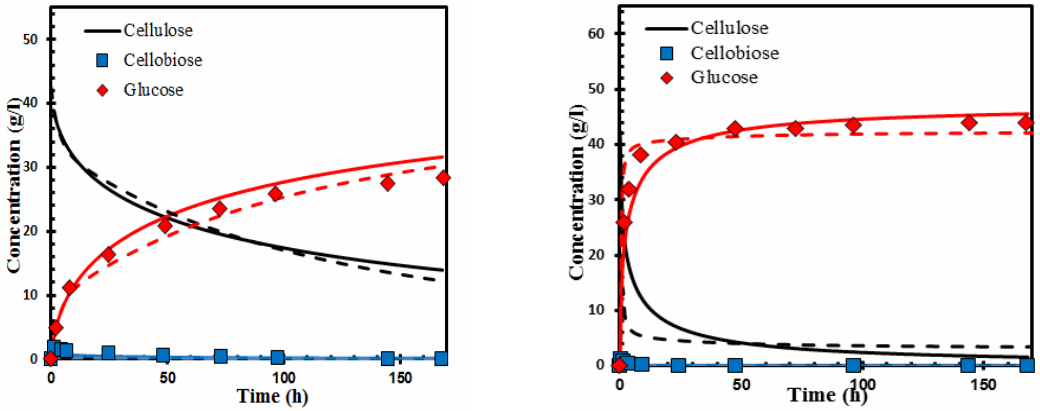

Figure 6: Comparison between predicted and experimental results for glucose, cellobiose and cellulose during enzymatic hydrolysis of cellulose at (a) 5 FPU/g-glucan; (b) 150 FPU/g-glucan enzymes loading (solid and dashed lines show the simulation results derived from Models 1 and 2 respectively)

In the study performed by Peri *et al.*,[19] enzymatic hydrolysis of non-crystalline cellulose (NCC) with an initial substrate concentration of 11.5 g/L and enzyme concentration of 1 and 3 FPU/g-glucan at 50 °C, pH 4.5, was investigated. A comparison between the predicted and the experimental results of enzymatic hydrolysis, based on the study of Peri *et al.*,[19] are shown in Figure 5. Also, Tables 1 and 2 list best-fit estimates of kinetic parameters. In addition, Zheng *et al.*[22] performed the enzymatic hydrolysis of pretreated creeping wild ryegrass, with an initial substrate concentration of 42.5 g/L, and enzyme concentration of 5 and 150 FPU/g-glucan, at 50 °C, pH 5. Cellulase activity of 90 FPU/mL corresponds to 54 mg protein/mL. A comparison between the predicted and the experimental results of enzymatic hydrolysis, based on the data of Zheng *et al.*,[22] are shown in Figure 6. Also, the estimated parameters from the two models are presented in Tables 1 and 2. As can be seen, the hydrolysis rate of cellulose depends on hydrolysis time and cellulase concentration. The hydrolysis rate increases with an increasing enzyme concentration. This behavior is demonstrated in Figure 5(a, b) and Figure 6(a, b). However, the hydrolysis rate did not increase with further increasing enzyme concentration, because of enzyme saturation on the surface of cellulose.

The sum of the squares of the weighted deviations has been calculated to be 11.65 and 5.49 for Models 1 and 2 in Figure 5, respectively. In addition, the values of $\chi 2(m)$ were 84.11 and 59.16 for Models 1 and 2 in Figure 6, respectively.

It can be observed that the predictions offered by both models fit well with the experimental values. However, the comparison of $\chi 2(m)$ values

shows that the modified model suggested in the present work is more accurate than the previously reported model in all the studied cases.

CONCLUSION

In the present study, a comparative analysis was performed between two models used to predict experimental results from enzymatic hydrolysis of microalgae and different cellulosic materials – a model reported in the literature (Zheng *et al.*) and another one modified in this work. The simulation results indicated that, as modified in the present work, the suggested model could successfully predict the production of glucose, cellobiose, and cellulose as a function of time during enzymatic hydrolysis. Comparing the predictions offered by the two models with the experimental results was carried out by comparing the values of $\chi^{2}(m)$. The results showed that the proposed modified model, with an average of $\chi^{2}(m)$ equal to 38.15, is more accurate than the previously reported model, with an average of $\chi^{2}(m)$ equal to 48.84.

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