

# Dilute acid hydrolysis of microalgal biomass for bioethanol production: an accurate kinetic model of biomass solubilization, sugars hydrolysis and nitrogen/ash balance

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**Abstract** In this paper, acidic hydrolysis (0–5 vol%) was performed on *Chlorella vulgaris* biomass using a range of temperature (100–130 °C) and reaction time (0–60 min) with high biomass load (10%–100 g L<sup>-1</sup>), in order to characterize the kinetic of biomass solubilization, hydrolysis of sugars, proteins and ash release, and to verify the main divergences and similarities in relation to lignocellulosic biomass. More than 90% of the sugars present in the biomass was hydrolyzed and later satisfactorily fermented by *S. cerevisiae*. The inclusion of acid concentration in the kinetic model for biomass solubilization and sugar hydrolysis led to a modified Michaelis–Menten equation able to simulate efficiently the acidic extraction/hydrolysis data of all experimental runs. Main divergences in relation to lignocellulosics were related to higher reaction order and lower activation energy, revealing better susceptibility of microalgal biomass to acidic treatment. The proposed process is promising and can be easily scaled up at industrial level.

**Keywords** Sulfuric acid · Michaelis–Menten · Nutrient recycling · *Saccharomyces*

## Introduction

Biomass is a highly promising fossil substitute raw material for future biofuel application owing to several reasons: abundant feedstock availability and possibility to be produced in almost all regions of the world, avoiding competition with food

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The original version of this article was revised. The figures caption of Fig. 4, Fig. 5 and Fig. 6 was published incorrectly. The right figure captions is updated in the article.

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crops [1]. However, its chemical structure (lignocellulose) is complex and change significantly depending on the type. For this reason, several methods of pretreatment are applied to exploit it, for example, alkaline for coconut husk fiber [2], acidic for citric waste [3], hydrothermal for sugarcane straw [4] or biological for wood [5], becoming more complex and severe as a function of the polymeric structure recalcitrance.

A potential bio-refinery scheme, claiming an environmental gain thanks to carbon cycle and the use of renewables, aims at a controlled depolymerization of biomass which includes the following steps: (1) fractionation of biomass into biopolymers: cellulose, hemicellulose, starch and lignin; (2) depolymerization of the biopolymers and (3) transformation of the monomers into value-added products [1].

More recently, microalgae have been developed and proposed as a potential source of biomass, especially for biofuels applications, initially more devoted to biodiesel (lipid fraction) [6], but then focused also on ethanol [7], methane [8], hydrogen [9] and butanol [10]. Microalgae have a number of advantages in comparison to higher plants (i.e. lignocellulosic biomass), such as higher growth rates and less recalcitrance due to the absence of lignin and lower amount of cellulose and hemicellulose, being starch (microalgae) or glycogen (cyanobacteria) the main reserve-polysaccharides which are easily hydrolysable by acids [11, 12].

Kinetic studies using acidic pretreatment of lignocellulosic materials are reported in the literature, but for biomass solubilization only [1, 13–17]. For lignocellulosic materials, the acidic treatment is effective as pretreatment to remove hemicellulose and de-structure its three-dimensional arrangement, then helping the enzymatic hydrolysis of cellulose [7, 18]. Thinking of microalgae and cyanobacteria [19, 20], the potential to obtain a higher sugar hydrolysis rate by acidic hydrolysis is increased, but so far most studies on microalgal biomass were focused to determine the best condition of hydrolysis, i.e., to find the optimized value of temperature, time of reaction, acidic concentration and biomass load [7, 21–23]. This method is faster but at the same time, it is not efficient to evaluate the nature of biomass hydrolysis and/or degradation processes. Kinetic and thermodynamic information is necessary to be used efficiently in the models to correctly perform process simulation and plant design. In any case, the nature of microalgal biomass is completely different from lignocellulosic biomass and the models traditionally used cannot be suitable for microalgae, so that a model of acidic sugars hydrolysis based on microalgal biomass as substrate has not been developed yet.

In addition, mass balance calculations of nutrients needed, mainly nitrogen and phosphorous, influence the global process economics, because they represent the largest costs in cultivating algae for fuels. The recycling and life cycle assessment (LCA) of these nutrients are a must [24–26]. Acidic hydrolysis as a non-specific process promotes not only the release of sugars but also of the other substances present in the biomass (such proteins and ash), and this additional fraction can be useful in the liquor during yeast/bacteria fermentation and needs to be quantified for an accurate investigation to close the loop of nutrients in the process.

In this paper, the range of temperature (100–130 °C) and reaction time (0–60 min) using high biomass load (10%–100 g L<sup>-1</sup>) were investigated in order to characterize the kinetic of biomass solubilization, hydrolysis of sugars, proteins

and ash solubilization from *Chlorella vulgaris* biomass using thermochemical hydrolysis with diluted acid (0–5 vol%). Divergences and similarities with lignocellulosic biomass will be highlighted as well.

## Experimental

### Microalgal biomass and biochemical characterization

*Chlorella vulgaris* biomass powder was produced by Neoalgae® (Micro seaweed products B-52501749). The characterization included the determination of moisture (method 934.01), ash (method 942.05), protein (method 2001.11), lipid content (method 2003.05), carbohydrates and monomers (HPLC) [27].

### Acidic hydrolysis and analytical procedures

Acidic hydrolysis was performed with 10% of solids load (microalgal biomass), in autoclave (Autoclave vapour-line<sup>eco</sup> VWR), using temperatures between 100 and 130 °C ( $P \sim 1$  atm), and changing the concentration of catalyst ( $H_2SO_4$ —98% Sigma®) (0, 1, 3 and 5 vol%) and the reaction time (0–60 min) in order to validate the kinetic model proposed and posteriorly discussed.

The mass yield (MY) of the process was evaluated on a dry weight basis after the thermochemical treatment by gravimetry using cellulose acetate filters of 0.45  $\mu m$  (Whatman®) at 105 °C and 2 h. Filters were pre-dried for 10 min at 105 °C in order to remove any moisture. The relation between solubilized biomass and mass yield is given by:

$$[\text{Solubilized biomass}](\%) = \frac{\text{Initial biomass load}(\frac{g}{L}) - \text{Mass yield}(\frac{g}{L})}{\text{Initial biomass load}(\frac{g}{L})} \cdot 100 \quad (1)$$

The amount of total extracted sugars (TS) was determined by Anthrone method [28] and reducing sugars (monomers, RS) using the DNS method [3, 29].

The % of sugars extracted/hydrolyzed was calculated by:

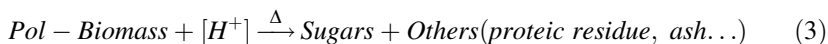
$$[\text{Sugar}](\%) = \frac{\text{Sugar concentration in the liquor}(\frac{g}{L})}{\text{Initial biomass load}(\frac{g}{L}) \cdot \text{Carbohydrates content}} \cdot 100 \quad (2)$$

Here the carbohydrate content is given as a relative value between 0 and 0.6 (generally the maximum of carbohydrate accumulation in microalgae and cyanobacteria is 0.6 = 60% of carbohydrate content, in dry weight basis) [7, 30, 31].

### Kinetic model

#### Reaction

The reaction of acidic biomass solubilization is commonly represented by:



The assumption is that  $H^+$  participates in the biomass solubilization and sugars hydrolysis as a catalyst, but interactions as reagent should be considered as well. In fact, some speculation about the effect of  $[H^+]$  only a catalyst was made, explained that it is possible of its neutralization for the biomass due the several biochemical fractions, mainly minerals [17].

### Mass yield

An  $n$ th order kinetics for biomass solubilization and an  $m$ th order for acid hydrolysis were used, resulting in an  $n + m$  overall reaction order. The acid concentration term was incorporated in the Arrhenius equation giving a modified equation:

$$\frac{dPol}{dt} = k[Pol]^n \quad (4)$$

Here Pol indicates biomass concentration, and where:

$$k = k_0[H^+]^m e^{\frac{-E_a}{RT}} \quad (5)$$

Here  $n$  and  $m$  are the orders of reactions to Pol and  $H^+$ , respectively.

The integration of Eq. 4 yields:

$$Pol^{1-n} - Pol_0^{1-n} = (n - 1)kt \quad (6)$$

Consequently:

$$Pol = (Pol_0^{1-n} + (n - 1)kt)^{\frac{1}{1-n}} \quad (7)$$

From Eq. 7, at constant Temperature ( $T$ ) and  $Pol$  biomass concentration it is possible to find the  $k$  values a corresponding to the  $[H^+]$  value applied. Then, using Eq. 5, since the values of  $T$  (K),  $R$  (J/(mol K)) and  $k$  ( $\text{min}^{-1} (\text{g/L})^{-n}$ ) are known, the values of  $m$  and  $E_a$  (kJ/mol) can be found.

### Total sugar concentration (extracted/hydrolyzed sugars)

The Michaelis–Menten model was applied to describe the total sugars extracted from the biomass, since sugars concentration in liquid-phase depends on the substrate concentration (biomass and acid concentration):

$$\frac{d Sugars}{dt} = k \quad (8)$$

The following composite constant is used here:

$$k = \frac{k_{\max[T,H^+]}[S]}{K_M + [S]} \text{ (Temperature and } [H^+] \text{ constant, i.e., only the time } (t) \text{ varies)} \tag{9}$$

When the substrate concentration is high, the equation becomes zeroth order [32]. In our work, it is proposed to express the [S] term by Eq. 9.1, including the proton as a reactant and the orders of reaction.  $K_M$  ( $(g/L)^{n+m}$ ) is the constant of half-saturation and  $k$  is the reaction rate constant ( $\text{min}^{-1}$ ). Parameter estimation was performed by non-linear regression validated by the least squares method [33].

$$[S] = [Pol]^n [H^+]^m \tag{9.1}$$

Thus, the final equation that can be obtained integrating Eq. 8 is:

$$[Sugar] \left( \frac{g}{L} \right) = \frac{k_{\max[T,H^+]} [Pol]^n [H^+]^m}{K_M + [Pol]^n [H^+]^m} t \tag{10}$$

Here  $t$  is the reaction time.

Additionally, selectivity (S) is defined as:

$$S = \frac{Y}{X} \tag{11}$$

Here  $Y$  represents the yield of the reaction and  $X$  the conversion of biomass ( $Pol$ ). The final reaction time studied in this work, 60 min was considered as the reference time.

### Protein and ash solubilization

The extents of solubilized protein and ash were calculated by:

$$[Solubilized\ component](\%) = \frac{Initial\ biomass\ load \left( \frac{g}{L} \right) \cdot Initial\ component\ content - Mass\ Yield \left( \frac{g}{L} \right) \cdot Final\ component\ content}{Initial\ biomass\ load \left( \frac{g}{L} \right) \cdot Initial\ component\ content} \cdot 100 \tag{12}$$

### Ethanolic fermentation

Fermentation was performed with *S. cerevisiae* (Cameo S.p.A.<sup>®</sup>) using the broth from acidic hydrolysis after adjustment the pH to 5.5 at 30 °C for 48 h. Reducing sugars were measured by DNS method and cellular growth by dry weight (described in the [Acidic hydrolysis and analytical procedures](#) section). Ethanol was determined by gas chromatography (Shimadzu GC-14A) injecting 5 μL of centrifugated at 5000 rpm per 10 min (Labnet, Spectrofuge 7 M, bought from Sigma–Aldrich, Milan, Italy) in Eppendorf<sup>™</sup>, with a Poropak QS packed column (80/100 mesh, 1.7 m length). The carrier gas was helium with a flow rate of 20 mL/min. The

injector, column oven and the detector temperatures were 150, 150 and 170 °C. The composition was computed from the GC peak areas according to a calibration curve.

Conversion factors are calculated by:

$$Y_{X/S} = \frac{\Delta X}{\Delta \text{Sugars}} \quad (13)$$

$$Y_{E/S} = \frac{\Delta \text{Ethanol}}{\Delta \text{Sugars}} \quad (14)$$

$$Y_{X/E} = \frac{\Delta X}{\Delta \text{Ethanol}} \quad (15)$$

Here  $\Delta$  is referred to the difference between time 0 and the end of fermentation, sugars indicate reducing sugars and X is the yeast concentration ( $\text{g L}^{-1}$ ).

Process and fermentation yield were calculated by:

$$\text{Yield}_{\text{Process}} (\%) = \frac{\Delta \text{Ethanol}}{0.5111 \text{ Initial Sugar}} \cdot 100 \quad (16)$$

$$\text{Yield}_{\text{Fermentation}} (\%) = \frac{\Delta \text{Ethanol}}{0.5111 \Delta \text{Sugar}} \cdot 100 \quad (17)$$

Here 0.5111 is the glucose-ethanol conversion factor according to the stoichiometry of Gay-Lussac.

## Results and discussion

The combined use of temperature, acid concentration and time was applied in order to evaluate the susceptibility of microalgal biomass to promote solubilization and, in particular, sugars extraction and hydrolysis, in order to propose a suitable model to simulate efficiently the process. Protein and ash extraction in the liquid phase were taken with account, as acid as a catalyst is not specific. Lastly, ethanolic fermentation was carried out to validate the fermentability of the hydrolyzed matter with *S. cerevisiae*, and a mass balance of industrial process flowsheet was proposed.

### Biomass characterization

The biochemical composition of *Chlorella vulgaris* is presented in Table 1. The biomass used has a relatively high content of proteins and ash, together with carbohydrate. It is important to remember that microalgae display a biochemical plasticity able to change their composition according to the nutritional and environmental factors. Specifically, for *Chlorella vulgaris*, nitrogen availability, residence time and light intensity allow to accumulate more or less carbohydrate in the biomass [30].

Carbohydrates in microalgae are present as cell wall components (generally cellulose and soluble hemicellulose) and plastids (mainly in the form of starch) [19].

**Table 1** Macrocomponents in *Chlorella vulgaris*

Components	% of dry weight			
	This study <i>Chlorella vulgaris</i>	[36] <i>Chlorella sp.</i> KR1	[37] <i>Chlorella sorokiniana</i>	[38] <i>Chlorella vulgaris</i> JSC-6
Protein	49.5 ± 0.29	16.6	–	–
Lipid	6.3 ± 0.15	38.0	–	–
Carbohydrates	23.0 ± 2.0	36.1	18.2	54
Glucose <sup>a</sup>	70.15	82.2	70.8	79
Xylose <sup>a</sup>	10.65	6.4	13.8	11.11
Arabinose <sup>a</sup>	10.91	5.54	–	9.25
Rhamnose <sup>a</sup>	5.73	5.26	6.5	–
Other <sup>a</sup>	2.56	–	1.2	–
Ash	7.18 ± 0.01	5.9	–	–
Moisture	5.41 ± 0.05	3.6	–	–
Other	8.61	–	–	–

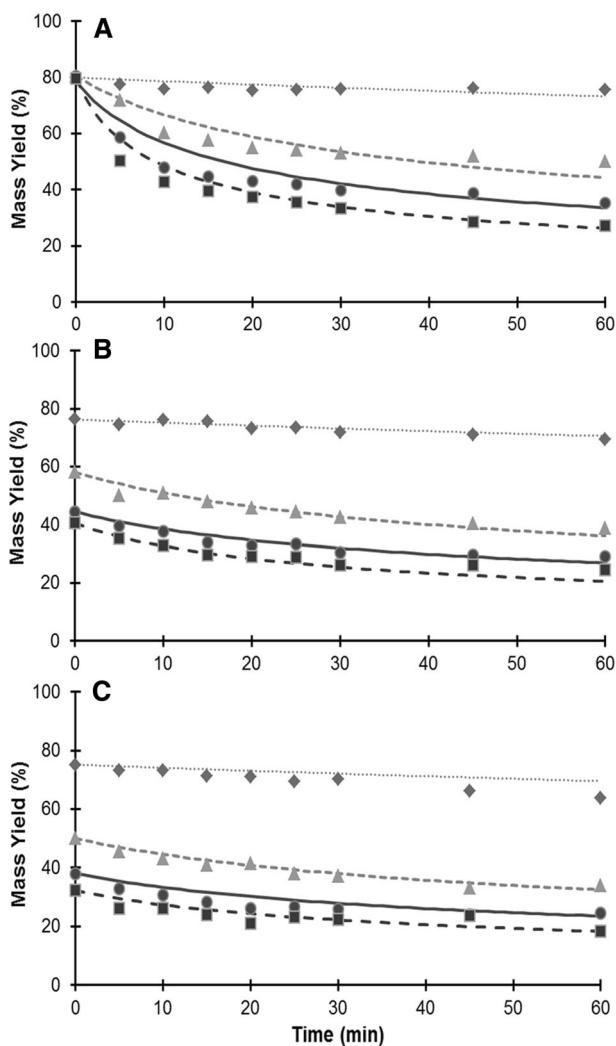
<sup>a</sup>% respect to the carbohydrate content

Glucose was found predominant monosaccharide in the biomass and accounts for more than 70% of total sugars, together with xylose (10.65%), arabinose (10.91%) and rhamnose (5.73%). These results to sugars composition in *Chlorella* are in according with literature data.

Xylose and arabinose are pentoses which cannot be fermented by *Saccharomyces*, the most widely used industrial microorganism, and this deserves to be considered as they represent more than 20% of total sugars in *Chlorella vulgaris*. Genetic improvement is needed in order to increase the ethanol tolerance and ability of yeast and bacteria species to ferment pentose [34]. In addition, some genders, as *Pichia*, *Candida* and *Kluyveromyces* are able to ferment pentose and hexose naturally but at lower rates in comparison to *Saccharomyces* [35]. However, this point was not addressed here because *S. cerevisiae* was used.

**Biomass solubilization**

As shown in Fig. 1, the range of temperature (110–130 °C) and acid concentration applied were efficient in the solubilization of microalgal biomass, reaching best values (around 20–25%) with 5% of acid and 60 min of reaction time. However, 120 °C and 3% of acid was considered the best option in terms of solubilization/acid ratio used. It is noteworthy that the zero point was considered when the temperature reached 110, 120 or 130 °C, i.e., the initial heating time was not included. Clearly, this had an effect in the experiments, as at zero point the initial biomass and sugar concentration are not 100% and 0, respectively (Fig. 1, but also Figs. 2 and 3 later on), but the effect is the same for all experimental runs and does not affect the kinetic model. Note that, even though most of published papers use less than

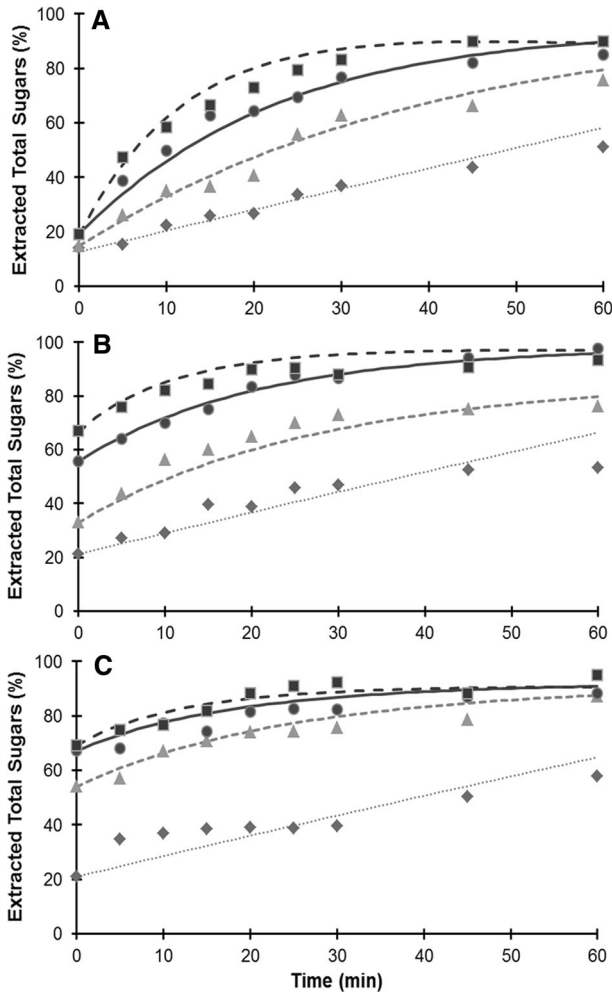


**Fig. 1** Mass yield versus time. **a** 110 °C, **b** 120 °C and **c** 130 °C. (filled diamond) 0, (filled triangle) 1 vol% (filled circle) 3 vol% and (filled square) 5 vol% of  $\text{H}_2\text{SO}_4$ . Lines represent model prediction. Standard deviation <3%

50 g  $\text{L}^{-1}$  of biomass [12, 23, 39], a biomass concentration of 100 g  $\text{L}^{-1}$  was chosen to obtain a suitable sugars concentration with respect to a real process, even though higher biomass concentration increases the viscosity and can disturb the saccharification yield [10, 22, 38].

According to Table 2, a high reaction order in comparison with lignocellulose solubilization was found: Average values were  $n = 3.6307 \pm 0.1818$  and  $m = 1.4161 \pm 0.0649$ . The activation energy of the process was  $E_a = 41.1919 \pm 0.0982$  kJ/mol. These values are much lower than cellulose/lignocellulosic biomass under acidic hydrolysis ( $n$  and  $m$  are first-order and  $E_a$  range

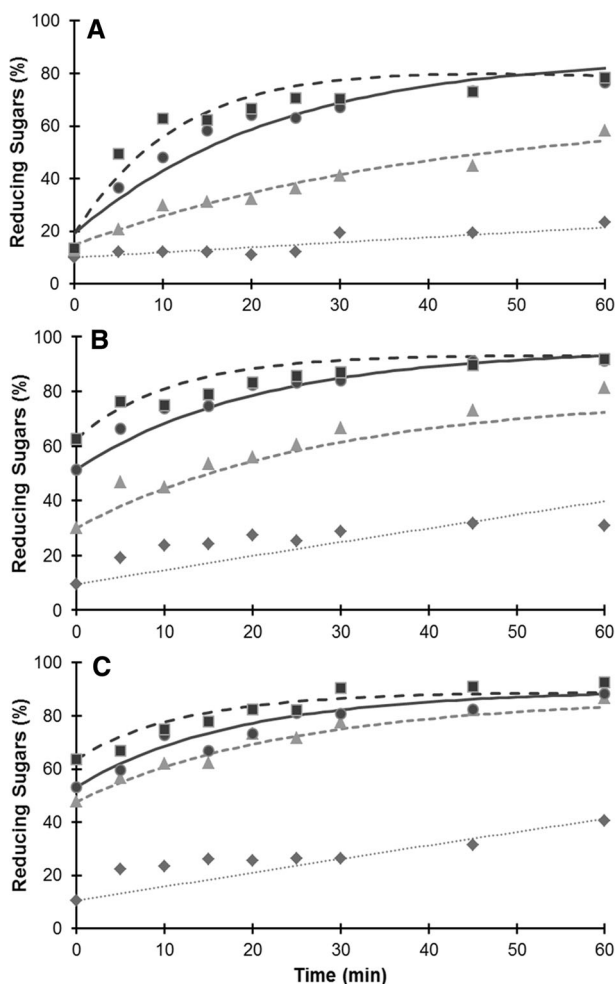




**Fig. 2** Total sugars versus time. **a** 110 °C, **b** 120 °C and **c** 130 °C. (filled diamond) 0, (filled triangle) 1 vol% (filled circle) 3 vol% and (filled square) 5 vol% of H<sub>2</sub>SO<sub>4</sub>. Lines represent model prediction. Standard deviation <4%

between 100 and 190 kJ/mol). Therefore, microalgal biomass has higher susceptibility to acidic hydrolysis, and the currently proposed process looks promising for application as a single step one to obtain fermentable sugars from microalgal biomass, i.e., using less energy. In the case of lignocellulosic biomass, the acidic treatment is used as pretreatment to remove the hemicellulose fraction and help enzyme accessibility to cellulose fraction (de-structuration of lignocellulose) [1], but additional cost due to the need of specific enzymes is one of the main bottlenecks to cellulosic ethanol consolidation.

The inclusion of the acid concentration in the kinetic of biomass de-structuration during a thermochemical hydrolysis is important and was already proposed [1, 13].



**Fig. 3** Reducing sugars (monomers) versus time. **a** 110 °C, **b** 120 °C and **c** 130 °C. (filled diamond) 0, (filled triangle) 1 vol% (filled circle) 3 vol% and (filled square) 5 vol% of H<sub>2</sub>SO<sub>4</sub>. Lines represent model prediction. Standard deviation <7%

The solubilization of lignocellulosic biomass is usually considered as first order with respect to biomass and acid concentration [13, 15]. This concept was also used in the case of microalgae [39, 40], but the same assumption was not verified in this work ( $n$  and  $m$ , Table 2), showing the importance of new development for an accurately scientific evaluation and application to process simulation and plant design.

Kinetic constant ( $k$ —(min<sup>-1</sup>) (g/L)<sup>- $m$</sup> ) values strongly increased as a function of temperature and acid concentration, being improved by 2–3 orders of magnitude with respect to the control condition (no acid addition). A similar effect of temperature is described in several works related to both lignocellulosic and

**Table 2** Kinetic constants at different temperatures to biomass solubilization during acidic hydrolysis

110 °C	Acid concentration (% v v <sup>-1</sup> )	k (reaction rate) (min <sup>-1</sup> ) (g/L) <sup>-m</sup>	n (reaction order)	SE (%)
Mass yield	0	$5.6161 \times 10^{-9}$	3.8741	1.2597
	1	$2.2092 \times 10^{-7}$	3.6457	3.8225
	3	$7.1992 \times 10^{-7}$	3.5578	3.1847
	5	$2.1592 \times 10^{-6}$	3.4454	3.4340
Ea activation energy (kJ/mol)			41.1013	
k <sub>0</sub> (min <sup>-1</sup> )			0.3682	
m (reaction order to H <sup>+</sup> )			1.3483	
R <sup>2</sup>			0.9993	
SE (%)			$1.38 \times 10^{-7}$	
120 °C	Acid concentration (% v v <sup>-1</sup> )	k (reaction rate) (min <sup>-1</sup> ) (g/L) <sup>-m</sup>	n (reaction order)	SE (%)
Mass yield	0	$5.7161 \times 10^{-9}$	3.8741	0.9519
	1	$3.3972 \times 10^{-7}$	3.6457	1.6721
	3	$1.0415 \times 10^{-6}$	3.5600	2.8233
	5	$3.3996 \times 10^{-6}$	3.4843	1.9537
Ea activation energy (kJ/mol)			41.2962	
k <sub>0</sub> (min <sup>-1</sup> )			0.3702	
m (reaction order to H <sup>+</sup> )			1.4221	
R <sup>2</sup>			0.9946	
SE (%)			$4.07 \times 10^{-7}$	
130 °C	Acid concentration (% v v <sup>-1</sup> )	k (reaction rate) (min <sup>-1</sup> ) (g/L) <sup>-m</sup>	n (reaction order)	SE (%)
Mass Yield	0	$5.8361 \times 10^{-9}$	3.8741	3.2611
	1	$4.3172 \times 10^{-7}$	3.6457	1.3024
	3	$1.4391 \times 10^{-6}$	3.5600	1.4737
	5	$4.1854 \times 10^{-6}$	3.4800	2.0706
Ea activation energy (kJ/mol)			41.1782	
k <sub>0</sub> (min <sup>-1</sup> )			0.3513	
m (reaction order to H <sup>+</sup> )			1.4778	
R <sup>2</sup>			0.9907	
SE (%)			$3.34 \times 10^{-7}$	

Average value ± Standard deviation

Ea = 41.1919 ± 0.0982 kJ/mol

k<sub>0</sub> = 0.3632 ± 0.0104 min<sup>-1</sup>

m = 1.4161 ± 0.0649

n = 3.6307 ± 0.1818

microalgal biomass [1, 12, 13, 22, 23], but so far has not been included at kinetic level, especially for microalgae.

### Extracted sugars (total sugars) and reducing sugars (monomers)

The extraction (total sugars) and hydrolysis (reducing sugars) were validated in the same ranges of temperature (110, 120 and 130 °C), acid concentration (0, 1, 3 and 5%) and reaction time (0–60 min) and are displayed in Fig. 2 and Clearly, at lower temperature (110 °C), the amount of total sugars is higher than the reducing sugars measured (Figs. 2a and 3a). In this case, it means that the sugars were separated from the biomass matrix but not efficiently converted to monomers. When the temperature increased (120 and 130 °C), total and reducing sugars values become closer to each other, thus extraction and hydrolysis can be considered as concomitant processes (extraction and hydrolysis) (Figs. 2b, 2c, 3b and 3c).

At 120 °C, more than 90% of reducing sugars were obtained when 3% of sulfuric acid and 30 min of reaction time were used. This was considered as the best condition in the range of the experiments performed. In fact, according to literature 90% of biomass was hydrolyzed when 50 g L<sup>-1</sup> of *Tribonema sp.* was submitted at 121 °C and 3% of sulfuric acid for 30 min [10]. Ashokkumar et al. [23] hydrolyzed 20 g L<sup>-1</sup> of *Scenedesmus bijugatus* biomass at 130 °C and 2% of acid, and obtained around 85% of saccharification. *Dunaliella tertiolecta* LB999 (50 g L<sup>-1</sup>) at 121 °C and 3.73% of sulfuric acid for 15 min provided a hydrolysis yield of 44.31%, but here time was probably limiting [41]. *Scenedesmus obliquus* (50 g L<sup>-1</sup>, 120 °C and 5% of sulfuric acid for 30 min) provided >90% of saccharification yield [22]. *Chlorella vulgaris* JSC-6 (120 g L<sup>-1</sup>, 121 °C and 4–6% of sulfuric acid for 20 min) [38], *Scenedesmus obliquus* CNW-N (10–40 g L<sup>-1</sup>, 121 °C and 1.5–2% of sulfuric acid for 20 min) [12] and *Chlamydomonas reinhardtii* (50 g L<sup>-1</sup>, 120 °C, 3% of sulfuric acid for 30 min) [39] reached a saccharification yield between 90 and 100%. All these optimized conditions from the literature agree with the experimental results obtained in our work, so that the presently developed model for *Chlorella vulgaris* could be assumed to be suitable for most of industrial microalgal species considered in the literature. In addition, no significant degradation processes were verified in the variables range used.

From Table 3, we note that most of extracted sugars was solubilized with higher acid concentrations (3 and 5%), but the kinetic constant did not increase as a function of temperature (see 120 and 130 °C with respect to 110 °C), probably due the reduced sugars content on the biomass, because >90% of sugars were already extracted (Fig. 2). As an example, with 5% of acid,  $k$  reduced from 1.612 to 1.391 and 1.378 at 110, 120 and 130 °C. On the other hand, kinetic constants for reducing sugars increased as a function of temperature and acid concentration in all experiments inside the range studied and more than 90% of hydrolysis was reached (Table 3; Fig. 3).

According to the assumption  $[S] = [Pol]^n[H^+]^m$ ,  $K_M$  can also be expressed including  $[H^+]$  in its expression, and a value of the half-saturation mass yield ( $Pol_{KM}$ ) can be calculated by:

**Table 3** Kinetic constants of sugar solubilization in acidic hydrolysis at different temperatures

110 °C	Acid concentration (% v v <sup>-1</sup> )	<i>k</i> (min <sup>-1</sup> )	SE (%)
Extracted total sugars	0	0.2000	2.8090
	1	0.5376	6.1345
	3	0.8312	12.2784
	5	1.6120	11.4015
120 °C	Acid concentration (% v v <sup>-1</sup> )	<i>k</i> (min <sup>-1</sup> )	SE (%)
Extracted total sugars	0	0.2101	0.9519
	1	0.6512	1.6721
	3	0.8613	2.8233
	5	1.3912	1.9537
130 °C	Acid concentration (% v v <sup>-1</sup> )	<i>k</i> (min <sup>-1</sup> )	SE (%)
Extracted total sugars	0	0.2130	3.0718
	1	0.6600	7.0591
	3	0.7513	3.3769
	5	1.3781	14.2573
110 °C	Acid concentration (% v v <sup>-1</sup> )	<i>k</i> (min <sup>-1</sup> )	SE (%)
Reducing sugars	0	0.0500	1.9692
	1	0.3276	2.1678
	3	0.7400	12.9241
	5	1.3815	6.8101
120 °C	Acid concentration (% v v <sup>-1</sup> )	<i>k</i> (min <sup>-1</sup> )	SE (%)
Reducing sugars	0	0.1003	5.2345
	1	0.5912	6.7966
	3	0.8915	4.6584
	5	1.3900	3.1307
130 °C	Acid concentration (% v v <sup>-1</sup> )	<i>k</i> (min <sup>-1</sup> )	SE (%)
Reducing sugars	0	0.1502	6.4838
	1	0.7008	3.4356
	3	1.1112	8.6658
	5	1.6120	4.4052
Acid concentration (% v v <sup>-1</sup> )			KM (g/L) <sup>n + m</sup>
0			10,557.0
1			268,920.5
3			556,314.8
5			764,521.5
Remembering: $KM = [Pol]_{KM}^n [H^+]^m$			
[Pol] <sub>KM</sub> (g/L)			42.02 ± 1.75

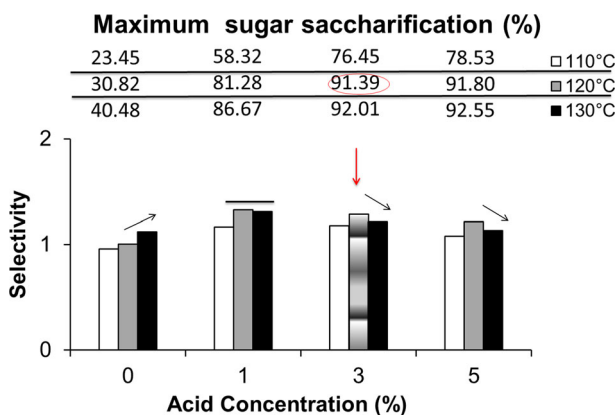
$$[Pol]_{KM} = \left( \frac{KM}{[H^+]^m} \right)^{\frac{1}{n}} \quad (18)$$

Interestingly, it was found that the value of  $Pol_{KM}$  is practically independent of acid concentration, with an average of  $42.02 \pm 1.75$  g/L (Table 3), thus the assumption made in the modified equation is reasonable. In this value of mass yield, around 80% of sugars were saccharified what justify the significantly reduction of the hydrolysis constant ( $k$ ), which to the model is half of the maximum ( $k_{max}$ ).

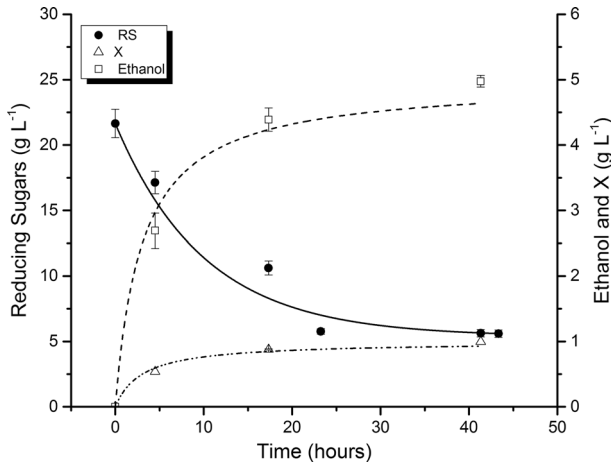
Furthermore, it is interesting to discuss selectivity values, as this variable allows us to evaluate the selection of best hydrolysis condition because it evaluates both the substrate ( $Pol$ ) conversion and the product yield (Hydrolyzed Sugars). By looking at Fig. 4, it is perceived that the selectivity increased as a function of acid concentration and temperature with 3% and 120 °C, where more than 90% of saccharification yield was obtained, confirming the best condition mentioned before.

### Ethanol fermentation

Fermentation of the hydrolyzed *Chlorella* biomass was carried out using *S. cerevisiae*, leading to the results presented in Fig. 5. Here, it can be seen that the fermentation was fast and reached a sugars consumption of 75% in 24 h. If we consider pentose-excluding sugars (hexoses) only, the reduction was 94.2%, indicating a good performance. The ethanol to sugar factor was 0.307, when the maximum stoichiometric value is 0.5111 (Gay-Lussac stoichiometry), but this is normal because a lower yeast inoculum concentration was used, and part of the carbohydrates are metabolized to cellular multiplication. Similar values are reported by other authors [2, 41, 42]. On the other hand, when a high inoculum concentration ( $10\% \text{ m v}^{-1}$ ) is used, this yield is increased to values about 95% of sugars



**Fig. 4** Selectivity versus acid concentration at different temperature. The maximum sugars recovery is reported above the graph to be correlate with the selectivity. The best condition was considered 3% of acid concentration and 120 °C, in evidence



**Fig. 5** Ethanolic fermentation from *Chlorella* hydrolyzed. RS, Reducing sugars and X, yeast concentration

**Table 4** Fermentation parameters

Experiment	% Sugars consumption	Final ethanol concentration (g/L)	Yield <sub>Process</sub> (%)	Yield <sub>Fermentation</sub> (%)	Y <sub>X/S</sub>	Y <sub>E/S</sub>	Y <sub>X/E</sub>
3% of acid at 120 °C for 30 min	74.2 (94.2) <sup>a</sup>	4.97 ± 0.09	45.0	60.6	0.080	0.307	0.250

<sup>a</sup> Excluding pentose, because 21.56% (Table 1) of sugars are pentose: xylose + arabinose (not naturally fermented for *S. cerevisiae*).  $\mu = 0.2311 \pm 0.03 \text{ h}^{-1}$

consumption, and more than 80% of ethanol yield can be achieved [43], whereas our work, it was 60.6% (Table 4).

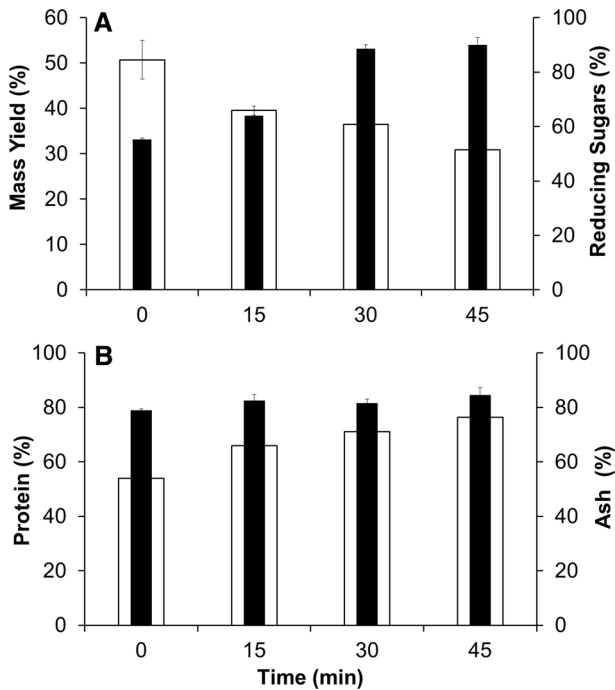
Literature values of ethanol/biomass yields are 0.163 g<sub>ethanol</sub>/g<sub>biomass</sub> (*Arthrospira platensis*—chemical hydrolysis) [42], 0.140 g<sub>ethanol</sub>/g<sub>biomass</sub> (*Dunaliella tertiolecta*—chemoenzymatic) [41] and 0.214–0.233 g<sub>ethanol</sub>/g<sub>biomass</sub> (*Chlorella vulgaris* FSP-E—enzymatic and chemical hydrolysis, respectively) [12]. In this work, a value of 0.150 g<sub>ethanol</sub>/g<sub>biomass</sub> was achieved, using low yeast inoculum concentration <0.1 g L<sup>-1</sup> of yeast and considering a biomass with 50% of carbohydrates). The stoichiometrically maximum value considering a biomass with 50% of carbohydrates is 0.26.

## Nutrient recovery and industrial process development

Acidic hydrolysis, as aforementioned, is a non-specific process and all biomass components are attached and can be dissolved. A special attention was paid to the protein content accounting for most of N fraction in the biomass and ash (mineral content, in particular P content), because N and P salts are the main nutrient requirements for microalgae cultivation (apart of carbon which comes from CO<sub>2</sub>), and consequently, they are crucial in determining cultivation costs.

The repetition of the experiments (with a 1 L—scale reactor) at 120 °C with 3% of acid (in quadruplicate) showed the same mass and saccharification yield reported before, confirming the reproducibility of the process (Fig. 6a). In view of ethanol production within a biorefinery approach, the acidic thermochemical process proved to be efficient in the solubilization of protein and ash fractions too: as shown in Fig. 6b, the liquid phase obtained in the best conditions (120 °C, 3% of acid and 30 min) contained around 80% of the ash and 70% of the proteins present in the initial biomass.

Nutrient recovery is a ‘hot topic’ in order to recycle the maximum of these nutrients in the process of microalgae cultivation so that to minimize the consumption of these nutrients [44–49]. This possibility was checked with respect to algae cultivation in several standard mediums, which are recipes providing an



**Fig. 6** Repetition of the acidic hydrolysis experiment at 120 °C using 3% of acid concentration. **a** Mass yield (White) and reducing sugars (Black). **b** Protein (White) and ash (Black) released in the liquid fraction (%)



optimized mixture of nutrients to support their growth [45]. In our currently proposed process, most of solubilized nutrients go to the fermentation, thus helping the yeast growth. After that, ethanol is distilled, yeast are centrifuged and recirculated, and the effluent remaining (stillage/vinasse) has the major concentration of nitrogen and phosphorous, among other nutrients. Therefore the recycling or recovery of this effluent is mandatory. In addition, the solid waste from hydrolysis process account for around 30% in mass of the initial biomass and its components can be recovered too. To this scope, the most efficient processes to recover the nutrients and increase energy efficiency are hydrothermal liquefaction (HTL), flash hydrolysis and anaerobic digestion.

For instance, HTL with *Arthrospira platensis* at 190–210 °C for 2–4 h was able to recover 78–90% of nitrogen present in biomass [50]. The main disadvantage of HTL is its high-energy duty, caused by the rather high temperature and pressure operating conditions. *Scenedesmus obliquus* was grown in the hydrolysate obtained by flash hydrolysis obtained from the same specie for flash hydrolysis at 280 °C and 9 s of residence time with a protein recovery of 65% in the liquid phase [46]. This species obtained better performance in batch cultivation than in the standard medium for autotrophic growth, thanks to the combination of heterotrophy (mixotrophy). In continuous cultivation mode, the productivities ranged between 0.62 and 0.72 g/(L day), showing satisfactory performances [26]. When anaerobic digestion is used for nutrient recycling, it is necessary to discuss about liquid phase (biofertilizer), because several non-gasifiable nutrients in the operation conditions, such as N and P, remain in mineral conditions in the final effluent, known as digestate, especially as ammonium and phosphate salts [47, 48]. Anaerobic digestion provides a liquid biofertilizer that can be also used for the cultivation of microorganisms (nutrient recycling) with an increase in sustainability and autonomy of the process; and is already integrated in an ethanol biorefinery [47]. For instance, *Chlorella vulgaris* was cultivated in sugarcane stillage anaerobically biodigested with the consumption of a great amounts of N and P of the effluent [49].

Economic and energetic analysis will confirm the real applicability of this technology because even with these promising numbers, cultivation costs of microalgae are still high in comparison with other crops [7]. Some points that need to be developed to increase the process feasibility are: solid fraction recycling, yeast recycling, stillage utilization, pentose fermentation and lipid fraction destination.

## Conclusion

In this work, it was shown that the acidic treatment of microalgal biomass is effective to solubilize biomass and hydrolyze the biomass sugars (by more than 90% present in *Chlorella vulgaris*). A model of  $n$ th order for biomass solubilization and a modified Michaelis–Menten equation were able to simulate efficiently the experimental hydrolysis data, acid concentration with  $m$ th order kinetics is included in this model, and showing an activation energy lower than for lignocellulosic biomass. After the thermochemical process, most of the proteins and ash present in the biomass are released in the liquid-phase and can be used in the ethanolic

fermentation step. It was also shown that *S. cerevisiae* fermented satisfactorily most of the sugars. The simplicity and efficiency of the process make this arrangement promising and a block flow diagram of an industrial process was eventually proposed.

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