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Abstract	Biofuel production using microalgae attracted much attention because it can be cultured using CO_2 and sunlight. With high carbohydrate content, microalgae have the potential to be used as a fermentation

feedstock for bioethanol production. In present work, chemo-enzymatic saccharification of *Chlorella sorokiniana* microalgae were investigated. Chemical hydrolysis of the biomass followed by enzymatic hydrolysis and was also evaluated the effect of combining the two enzymes and the sequential addition. The effect of α-amylase concentrations was analyzed in ranged between 50 and 8000 U/g of biomass and for amyloglucosidase between 90 and 600 U/g of biomass. The higher concentrations showed the highest conversion of reducing sugars. The α-amylase concentration 8000 U/g of biomass presented a conversion of 43.06 ± 2.92% (w/w), while amyloglucosidase with 600 U/g of biomass obtained 76.57 ± 6.42% (w/w). The combination of two enzymes simultaneously was more efficient than the sequential addition for low enzyme concentrations (α-amylase 50 U/g and amyloglucosidase 90 U/g) with a total reducing sugar of 22.78 ± 3.06 and 16.92 ± 2.06% (w/w), respectively. On the other hand, using the higher enzymes concentrations, no difference was observed between the two addition strategies, 58.9 ± 3.55 and 57.05 ± 2.33% (w/w) for the sequential and simultaneous, respectively. Both strategies didn't present advantage, since the amyloglucosidase enzyme alone produced slightly higher results. Even thought, the obtained results showed successfully performed saccharification of microalgal biomass and clearly point to microalgae use for saccharification and subsequent bioethanol production.

Keywords Hydrolysis - *Chlorella sorokiniana* - Bioethanol

Chemo-Enzymatic Saccharification Strategy of Microalgae *Chlorella Sorokiniana*

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Abstract. Biofuel production using microalgae attracted much attention because it can be cultured using CO_2 and sunlight. With high carbohydrate content, microalgae have the potential to be used as a fermentation feedstock for bioethanol production. In present work, chemo-enzymatic saccharification of *Chlorella sorokiniana* microalgae were investigated. Chemical hydrolysis of the biomass followed by enzymatic hydrolysis and was also evaluated the effect of combining the two enzymes and the sequential addition. The effect of α-amylase concentrations was analyzed in ranged between 50 and 8000 U/g of biomass and for amyloglucosidase between 90 and 600 U/g of biomass. The higher concentrations showed the highest conversion of reducing sugars. The α-amylase concentration 8000 U/g of biomass presented a conversion of 43.06 ± 2.92% (w/w), while amyloglucosidase with 600 U/g of biomass obtained 76.57 ± 6.42% (w/w). The combination of two enzymes simultaneously was more efficient than the sequential addition for low enzyme concentrations (α-amylase 50 U/g and amyloglucosidase 90 U/g) with a total reducing sugar of 22.78 ± 3.06 and 16.92 ± 2.06% (w/w), respectively. On the other hand, using the higher enzymes concentrations, no difference was observed between the two addition strategies, 58.9 ± 3.55 and 57.05 ± 2.33% (w/w) for the sequential and simultaneous, respectively. Both strategies didn't present advantage, since the amyloglucosidase enzyme alone produced slightly higher results. Even thought, the obtained results showed successfully performed saccharification of microalgal biomass and clearly point to microalgae use for saccharification and subsequent bioethanol production.

Keywords: Hydrolysis · *Chlorella sorokiniana* · Bioethanol

1 Introduction

Algae are the primary producers of oxygen in the aquatic environment. These microorganisms are widely distributed in marine systems and have a great diversity with respect to size, morphology, life cycle, pigments and metabolism. About half of the global oxygen production is accomplished by marine microalgae. They play an important role in CO_2 recycling through photosynthesis, which is similar to higher plants in O_2 [1]. In addition to having a long history of use as food and as live feed in aquaculture, microalgae have also been considered as a promising source for products for industrial applications, such as pharmaceutical, cosmetics or biofuels [2].

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Microalgae have been considered the third generation as feedstock for biofuel production based on the expectation that large amounts of biomass will become available at an acceptable cost. Biofuel production using microalgae has attracted much attention because it can be cultured using CO_2 and sunlight [1]. The carbohydrates composition of microalgae is mainly polysaccharides which are entrapped in the cell walls and between the intercellular matrices. The monosaccharide components include glucose, mannose, ribose/xylose, rhamnose, and fucose. The carbohydrate composition varies between microalgae strains and up to 70% dry weight of microalgae has been reported [3]. Also, cellulose is reported as the main structural component of the cell wall of most microalgae species. With the high carbohydrate content, microalgal biomass has the potential to be used as a fermentation feedstock for bioethanol production. The production of bioethanol from biomass involves the following process steps: biomass pretreatment, saccharification, fermentation into bioethanol and product recovery. Biomass pretreatment is also a necessary stage to increase the surface area, to enhance sugars solubility and to improve substrate digestibility. Pretreatments have been viewed as one of the most crucial and expensive processing stages in biomass conversion to fermentable sugars, as a preliminary stage to produce bioethanol [4]. These can be physical, chemical or enzymatic and can be combined to disrupt and break down complex carbohydrates [4]. Saccharification is one of the most crucial steps as fermentable sugars such as glucose and mannose are released and metabolized in the presence of yeast to produce bioethanol [5]. Different enzymes are used in the hydrolysis step and the process is influenced by numerous factors including cellulose crystallinity, substrate surface area, cell wall thickness, porosity, mass transfer, and hemicellulose or lignin contents [6]. Since microalgae have been reported to have no lignin composition, it can be categorized as a cellulosic based material allowing development of a cost-effective processes. In the present work, is used the green microalgae *Chlorella sorokiniana*, a robust industrial species, due to their fast growth rates and simple cultivation requirements under typical conditions and tolerant to high temperatures and levels of solar irradiance [7]. Our goal is to hydrolyze the carbohydrates present in the biomass of the microalgae *Chlorella sorokiniana*, in simple sugars, so that they can be fermented for bioethanol production.

According to the results obtained in Hernández et al. work [4], the highest concentration of monosaccharides was achieved by the combination of acid pretreatment and enzymatic hydrolysis to enhance complex carbohydrates break down into simple sugars. As Hernández et al. [4] and Lee et al. [5], proposes the combined use of chemical hydrolysis with enzymatic hydrolysate, as we will develop this strategy in the present work. What distinguishes us from these works is the optimization of the enzyme concentrations, before the combination of the two, in the optimum concentrations and in the conditions with the use in less amount of enzyme. We chose to use the combination of enzymes two forms, simultaneous and sequential. Not all the bibliography in the area reports the need to use two steps for hydrolysis in fermentable sugars. Some papers report only the use of acid hydrolysis, as Ngamsirisomsakul et al. [8] suggested the use of sulfuric acid as suitable for biomass hydrolysis. For Kim et al. [9], sulfuric acid hydrolysis was more efficient than enzymatic treatment with pectinases, amylases, and cellulases for the microalgae C. vulgaris while, Shokrkar et al.

[10], reports the use only the enzymatic hydrolysate with the simultaneous use of cellulases, amylases, amyloglucosidase.

To achieve our goal, the enzymatic hydrolysis of *C. sorokiniana* microalgae was carried out, using two enzymes, α-amylase and amyloglucosidase. The way of adding them, whether sequentially or simultaneously, was evaluated. Previously the algal biomass underwent acid pretreatment.

2 Materials and Methods

2.1 Microalgae and Culture Conditions

The microalgae *Chlorella sorokiniana* 211-32 [7] was obtained from the culture collection of the Institute of Plant Biochemistry and Photosynthesis, IBVF, (Seville, Spain). Microalgae was cultivated in 1M tris-acetate-phosphate medium [11] pH = 7.2, grown under continuous white light irradiation of 100 μE $m^{-2}s^{-1}$ and aerated at 25 °C. The scale up of the microalga is made from 20 mL up to 3 L, with 20% (v/v) inoculum between scales. Growth is monitored by optical density at λ = 750 nm (GBC DBUV instrument Cintra 202, Australia). The microalgal biomass was harvested at the end of the exponential phase using a centrifuge (Hettich Zentrifugen Universal 320, Germany) at 2934 G for 5 min and then freeze dried.

2.2 Chemical Hydrolysis

Autoclave hydrolysis assays were performed using microalgal biomass suspended in 4% (v/v) H_2SO_4 solution to set a concentration of 50 g of lyophilized biomass/L and autoclaved at 121 °C for 30 min [4]. For further studies of enzymatic hydrolysis and sugar content analysis the pH of the hydrolysate was adjusted with NaOH solution.

2.3 Enzymatic Hydrolysis

Enzymes. The enzymatic saccharification of *Chlorella sorokiniana* was conducted using commercial enzymes α-Amylase from *Aspergilus oryzae* powder 30 U/mg (10065-50G) and Amyloglucosidase from *Aspergillus niger* 300 U/mL (A7095-50ML) were purchased from Sigma Aldrich.

α-Amylase Hydrolysis. Different α-amylase concentrations of 50, 500, 5000 and 8000 U/g of biomass were tested, in order to find the highest total reducing sugar content. The hydrolysis optimum conditions were pH 5.5 at 95 °C for 3 h. All the different concentrations were performed in triplicate.

Amyloglucosidase Hydrolysis. Different concentrations of amyloglucosidase (90, 240, 300 and 600 U/g of biomass) were tested in order to evaluate the higher reducing sugar content. The hydrolysis optimum conditions were pH 4.5 at 55 °C for 3 h and all the different concentrations tested were performed in triplicate.

Simultaneous and Sequential Hydrolysis. To test the influence of the combined use of the two selected enzymes, two addition strategies were designed. One sequential and one simultaneous. For sequential addition strategy, first α-amylase at the concentrations of 50 and 8000 U/g was added and then amyloglucosidase at concentrations 90 and 600 U/g. In summary, the sequential use of the two enzymes was tested by combining the two lowest and highest enzyme concentrations, in two separate trials. The optimum conditions of each of the enzymes were used, as described in 2.3. To simultaneous hydrolyzing assay, the combination was also tested for the combination of the two less and more concentrated enzymes, as in the sequential assay. In this case, the enzymes were added at same time and the test conditions were adjusted to pH = 5 and T = 60 °C for 6 h, defined conditions based on optimal amylase and amyloglucosidase conditions.

Figure 1 shows a graphical diagram of the defined strategy of enzymatic hydrolysis.

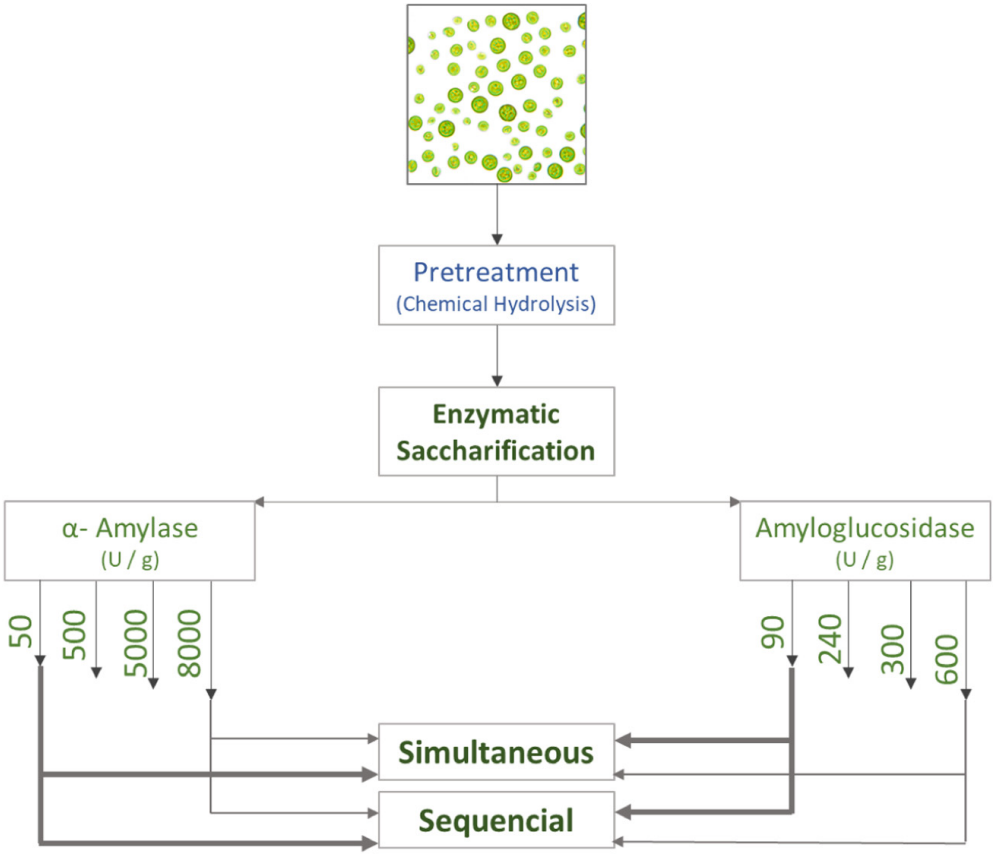

Fig. 1. Graphical diagram of the enzymatic hydrolysis strategy.

2.4 Analytical Procedures

Samples after the pretreatment and the different enzymatic hydrolysis were taken to analyze the content in reducing sugars. Reducing sugars were measured using the dinitrosalicylic acid (DNS) method with glucose as a standard [12].

The total reducing sugars content were calculated as:

$$\textit{Total reducing sugar content}\ (\%) = \frac{\text{Reducing sugar}\ (g/l)}{\text{inicial dried biomass}\ (g/l)}.100 \qquad (1)$$

2.5 Statistical Analysis

All experiments were carried out in triplicate and the results were the mean of six values (three replicates of the process and two replicates of the analysis) and the data were expressed as the mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to examine the difference among individual treatment and optimum condition. GraphPad Prism version 8.1.0 (GraphPad Software, Inc., USA) was used for all statistical analysis.

3 Results and Discussion

Generally, hydrolysis of carbohydrates involves liquefaction and saccharification steps. In order to saccharify the intracellular carbohydrates, such as starch granules [13] and the cell wall carbohydrates, the microalgae cell wall needs to be broken down by a pretreatment. However, in our case, the autoclaving pretreatment step, with microalgae diluted in aqueous solutions, did not affect the saccharification efficiency (data not showed). To enhance the saccharification efficiency, the saccharification experiments were performed, with a dilute acid pretreatment combined with a autoclaving step, before enzymatic hydrolysis of the microalgae biomass. So, in these studies, all enzymatic hydrolysis were performed after pretreatment by autoclaving with acid hydrolysis at 4% (v/v) sulfuric acid [4], denominated as chemical hydrolysis (CH). The results obtained by our group, was concordant with the results of Hernandez et al. [4], also proved the effectiveness of the combination of acid pretreatment and autoclaving, to enhance complex carbohydrates break down into simple sugars, to be used in the bioethanol production process from microalgal biomass.

The followings sections showed different results of chemo-enzymatic hydrolysis optimizations and strategies to maximized fermentable sugars for bioethanol production studies.

3.1 Optimization of α-Amylase Concentration After Acid Hydrolysis

The constitution of the cell wall varies depending on the type of microalga, being difficult to finding an optimal concentration of α-amylase in the literature. The scanning of various concentrations is an interesting study and poorly reported. It is known that α-amylases can degrade α−1, 4-glycosidic bonds of raw starches; therefore, α-amylases are among the most important raw starch-degrading enzymes [14].

To find the best concentration of the α-amylase enzyme, were tested different concentrations, some of which are already described in the literature [10, 15, 16]. The concentrations tested was 50, 500, 5000 and 8000 U/g (Fig. 2) and the assay was

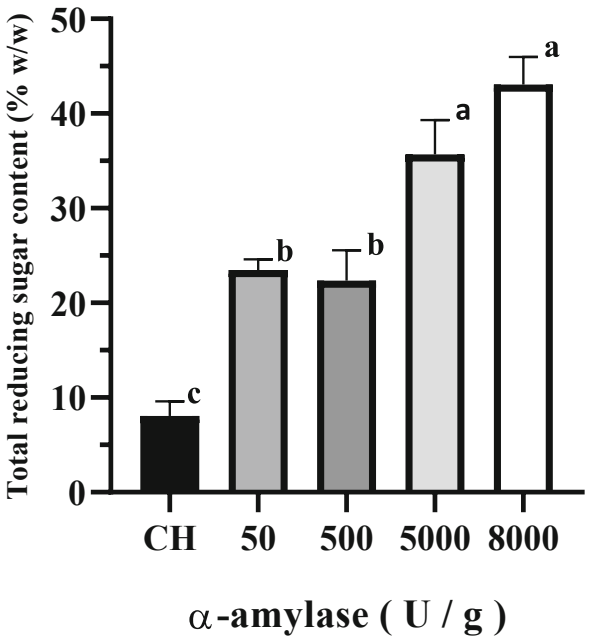

Fig. 2. Chemo-enzymatic saccharification of Chlorella sorokiniana at 50 g/L lyophilized biomass. Chemical hydrolysis (CH): 4% H_2SO_4 (v/v) at 121 °C autoclaving 30 min. Enzymatic Hydrolysis at different α-amylase concentration (50, 500, 5000 and 8000 U/g of biomass). The values are average and standard deviation of 3 different experiments. The statistical significance of the results was evaluated by one-way ANOVA using GraphPad Prism 8.1.0.

performed with optimum conditions for the performance of the α-amylase enzyme, pH 5.5 and temperature 95 °C for 3 h.

Figure 2 shows the total reducing sugar for chemical hydrolysis, 4% H_2SO_4 (v/v) at 121 °C autoclaving 30 min and for different tested α-amylase concentrations, 50, 500, 5000 and 8000 U/g of biomass. For CH were achieved 8.1% (w/w) and for 50, 500, 5000 and 8000 U/g of α-amylase concentration, the obtained values were 23.48 ± 1.13, 22.36 ± 3.21, 35.67 ± 3.70 and 43.06 ± 2.92% (w/w), respectively. For the α-amylase concentration of 50, 5000 and 8000 U/g biomass, there was an increase in sugar content of 1.9, 3.4, 4.3 times in relation to pretreatment.

The results shown significant differences of the pretreated biomass with the chemical hydrolysis and the different enzyme concentrations. It has been found that the lower concentration will not be enough to obtain the highest total reducing sugar content. The used of 8000 U/g of α-amylase exhibited the best saccharification efficiency after 3 h of reaction time (Fig. 2). Considering the results obtained from the selected enzyme concentrations, we can verify that the fact that Shokrkar et al. [10] used a mixed culture of algae and in our study, we focus in only one microalga, this point has an impact on the obtained results. Shokrkar et al. [10] using 50 U/g of α-amylase during 12 h obtained 19% and we with our study, in 3 h we obtained 23.48 ± 1.13% (w/w). We proved, at least, in our case with the strain *Chlorella sorokiniana* that less time can produces similar sugar content. Testing a higher enzyme concentration allows to accomplish higher sugar content. All these came to show the need to do more extensive studies and adapted the algae to use.

The α-amylase concentration (8000 U/g) described by Ometto et al. [15] showed the highest percentage of fermentable sugars of *Chlorella vulgaris*, although not significantly different from the 5000 U/g described by Marsalkova et al. [16] with the microalga *Chlorella* sp.. Thus, it is possible to reinforce the above-mentioned on the adaptation of the hydrolysis conditions to each species of algae and even to realize that, even among similar species the results in terms of available sugars may not be the same. Clearly, adaptation of the enzyme concentration to the species does not only apply to the α-amylase enzyme, but to the remaining enzymes used in microalgae hydrolysis, such as the second enzyme used in this work, amyloglucosidase, that will be discussed in next section.

3.2 Optimization of Amyloglucosidase Concentration After Acid Hydrolysis

Similar to the α-amylase enzyme assay, some of the tested amyloglucosidase concentrations were found in the literature [5, 6]. The enzyme concentrations were 90, 240, 300 and 600 U/g of biomass in order to obtain a scan of different concentrations of amyloglucosidase for the microalgae *Chlorella sorokiniana*. The assay was performed with optimum conditions for the functioning of the α-amylase enzyme, pH of 4.5 and temperature 55 °C for 3 h. The function of amyloglucosidase is hydrolyze α−1, 4 and α−1, 6 glucosidic bonds of oligosaccharides into glucose [17].

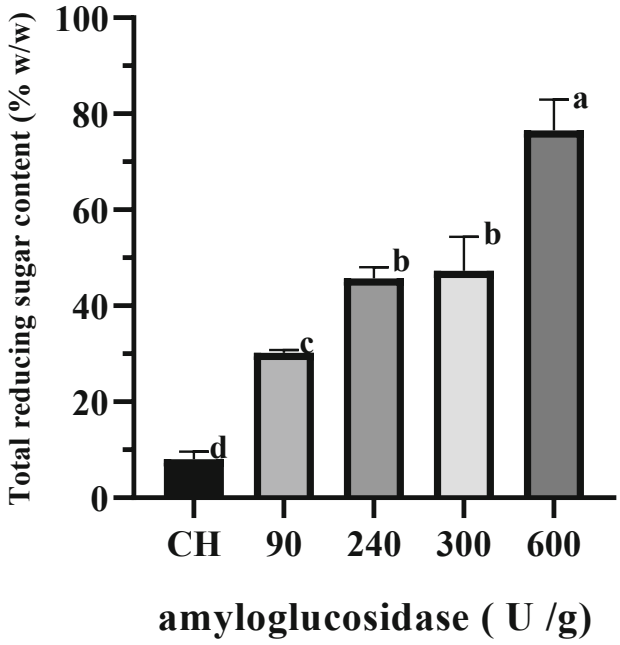

Fig. 3. Chemo-enzymatic saccharification of *Chlorella sorokiniana* at 50 g/L lyophilized biomass. Chemical hydrolysis (CH): 4% H_2SO_4 (v/v) at 121 °C autoclaving 30 min. Enzymatic Hydrolysis at different amyloglucosidase concentration (90, 240, 300 and 600 U/g of biomass) under pH 4.5, 55 °C, 3 h. The values are average and standard deviation of 3 different experiments. The statistical significance of the results was evaluated by one-way ANOVA using GraphPad Prism 8.1.0.

Figure 3 shows the total reducing sugar for chemical hydrolysis, 4% H_2SO_4 (v/v) at 121 °C autoclaving 30 min and for different tested amyloglucosidase concentrations, 90, 240, 300 and 600 U/g of biomass. For CH were achieved 8.1% (w/w) and for 90, 240, 300 and 600 U/g of amyloglucosidase concentration the obtained values were 30.21 ± 0.60, 45.70 ± 2.35, 47.32 ± 7.09 and 76.57 ± 6.42% (w/w), respectively. As showed in Fig. 3, significant differences of the pretreated biomass with the chemical hydrolysis and the different enzyme concentrations were verified. The lower amyloglucosidase concentration, 90 U/g biomass, had the lowest reducing sugar content and the highest concentration, 600 U/g, the highest reducing sugar content. Lee et al. [3] in studies with the microalgae *Chlorella* sp., in one hour of enzymatic hydrolysis using 240 U/g of amyloglucosidase at same conditions, achieve 37.9% of reducing sugar. In our studies, increasing the hydrolysis time from 1 to 3 h, for all enzyme concentrations, a higher concentration of fermentable sugars was achieved.

The strategy of using amyloglucosidase concentrations higher than those described in the literature ($\geq$ 240 U/g of biomass) found to be very advantageous and resulted in a significant increase of reducing sugar content. The action of the enzyme has a positive effect on the hydrolysis of the algal biomass, presenting an increase in the content of sugars relative to the CH. For the amyloglucosidase concentration of 90, 240 and 600 U/g biomass, there was an increase in sugar content of 3.7, 5.7, 9.5 times in relation to pretreatment. The concentrations of the amylase and amyloglucosidase enzymes were optimized, presenting high sugar conversions. To maximize conversion to fermentable sugars, different hydrolysis strategies were discussed in the following section.

3.3 Simultaneous and Sequential Use of α-Amylase and Amyloglucosidase

Since the isolated use of the amyloglucosidase and α-amylase enzymes showed promising results in obtaining hydrolysates with high reducing sugar content, i.e. fermentable sugars for bioethanol production, a final assay combining the two enzymes was designed to maximize more the hydrolysis yield. Was evaluated the influence of the hydrolysis strategy, a sequential and a simultaneous additions, using the two previously optimized enzymes, four experiments were carried out, two with the lowest enzyme (Fig. 4 – case 1) concentrations and two with the highest concentrations (Fig. 4 – case 2).

The addition of the enzymes was differentiated, using the optimal conditions of each enzyme. The enzymes were sequentially added, initially α-amylase and after 3 h, the amyloglucosidase was added. In the second case, the enzymes were added simultaneously, using intermediate operating conditions favorable to the two enzymes. In both cases, the reaction time of the enzymes was 6 h and this reaction was preceded by a chemical hydrolysis. The results are shown in Fig. 4 and for sequential and simultaneous 1 the total reducing sugar content were 16.92 ± 2.06 and 22.76 ± 3.06% (w/w), respectively. For the second case, higher enzyme concentrations, 58.90 ± 3.55 and 57.05 ± 2.33% (w/w) of total reducing sugar content were achieved for the sequential and simultaneous, respectively.

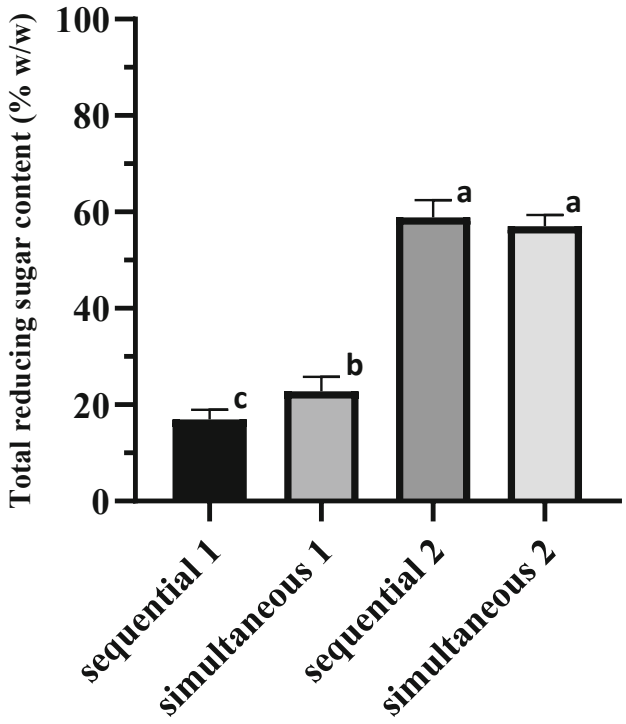

Fig. 4. Chemo-enzymatic saccharification of *Chlorella sorokiniana* at 50 g/L lyophilized biomass. Chemical hydrolysis (4% H_2SO_4 (v/v) at 121 °C autoclaving 30 min, followed by one of next conditions. Sequential 1: 50 U/g of α-amylase under pH 5.5, 95 °C for 3 h and amyloglucosidase at 90 U/g of biomass under pH 4.5, 55 °C for 3 h. Simultaneous 1: 50 U/g of α-amylase and amyloglucosidase at 90 U/g of biomass under pH 5.5, 65 °C for 6 h. Sequential 2: 8000 U/g of α-amylase under pH 5.5, 95 °C for 3 h and amyloglucosidase at 600 U/g of biomass under pH 4.5, 55 °C for 3 h. Simultaneous 2: 8000 U/g of α-amylase and amyloglucosidase at 600 U/g of biomass under pH 5.5, 65 °C for 6 h. The values are average and standard deviation of 3 different experiments. The statistical significance of the results was evaluated by one-way ANOVA using GraphPad Prism 8.1.0.

Significant differences were observed between simultaneous and sequential addition in the first case, for low enzyme concentration. However, no differences were observed in the second case, with high enzyme concentration. For low enzyme concentrations, the combination of two enzymes simultaneously, after the acid treatment, was more efficient than the sequential addition, first α-amylase followed by the addition of amyloglucosidase. These results are in agreement with the reported by Shokrkar et al. [10], who describes that the simultaneous addition of these enzymes increased the rate of sugars production, with 20% of reducing sugar content for a 12 h of microalgae enzymatic hydrolysis. Our sugar content is similar and was obtained in less time, only 3 h. The authors Shokrkar et al. [10] work only with low enzyme concentrations, and it is not possible to predict the results for higher enzyme concentrations. So, we tested and verified that with higher concentrations, the second case, that there were no significant differences between the two addition strategies, being the values very similar, 58.90 ± 3.55 and 57.05 ± 2.33% for the sequential and simultaneous, respectively. The results obtained for the highest concentrations of enzymes, 8000 U/g of α-amylase and 600 U/g of amyloglucosidase, both in sequential and simultaneous addition, are

quite promising. However, using only amyloglucosidase gives similar sugar conversion values (Fig. 3), which leads us to question. One of the possible causes, for this similarity, may be that the enzyme amyloglucosidase in higher concentrations, is more susceptible to the operating conditions, since in the simultaneous 2, the operating conditions was a compromise between the optimal conditions for the two enzymes. Another explanation, could be related to the presence of α-amylase enzyme at high concentrations and possible competition for the substrate between the two enzymes, leading to slightly lower content. In any case, the results obtained are quite promising, with content in reducing sugars higher than or quite similar to those described in the literature. We optimize enzyme concentrations (poor data found in the bibliography), hydrolysis time and different strategies of their use. It has also been possible for us to conclude that adaptation to the alga strain to be used may be important in hydrolysis studies.

With this work pave the way for the ultimate goal, that will be the fermentation of the sugars from the hydrolysate of the microalga *C. sorokiniana*. The fermentation will be pertinent to implement a Simultaneous Saccharification and Fermentation (SSF) strategy. Alternatively, the sugars, already obtained with very promising reducing sugar contents, can also be fermented in an isolated step of fermentation to produce ethanol (SHF).

4 Conclusions

In the study, with the addition of enzymes separately, the two maximum concentrations showed the highest conversion of reducing sugars, with a previously chemical hydrolysis. The 8000 U/g of α-amylase concentration presented a conversion of 43.06 ± 2.92% (w/w), while 600 U/g of amyloglucosidase had a conversion of reduced sugar by g biomass of 76.57 ± 6.42% (w/w). For low enzyme concentrations, the combination of two enzymes simultaneously, after the acid treatment, was more efficient than the sequential addition, where α-amylase was added followed by amyloglucosidase, with reducing sugar content of 22.76 ± 3.06 and 16.92 ± 2.06% (w/w), respectively. The addition sequential and in simultaneous present similar results, 58.90 ± 3.55 and 57.05 ± 2.33% (w/w), respectively with high release of fermentable sugars. For the higher concentrations of enzyme there was no advantage in the use of the two enzymes in these two addition strategies, since the amyloglucosidase enzyme only produced slightly higher results. Even thought, the obtained results from saccharification of microalgal biomass was successfully performed and clearly showed that biomass of microalgae could be used for saccharification and subsequent bioethanol production.

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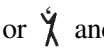
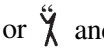
Insert single quotation marks	(As above)	 or  and/or  or 
Insert double quotation marks	(As above)	 or  and/or  or 
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