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USE OF mCLEAs OF GLYCOSIDASES TO HYDROLYSE MICROALGAL EXTRACTS

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1. INTRODUCTION

Overuse of fossil fuels during the last decades has been the cause of the current problems of global warming on earth. In addition, it has also created a heavy dependence towards a single and non-renewable source of energy (Goldemberg, 2007). In order to overcome such issues, biofuels propose an ecological solution, being a renewable, non-polluting, biodegradable and socially-accepted alternative. One of the main criticisms that biofuels originally drew was fermenting foodstuff, as maize starch or sugarcane bagasse, for bioethanol production (Rathmann *et al.*, 2010). However, new options for biofuel production not subject to these complications arouse, including lignocellulosic biomass (Somerville *et al.*, 2010) or microalgal cultures (Wijffels and Barbosa, 2010).

Microalgae prove an effective source of biofuels due to their numerous advantageous features. These include little land area for large cultures and adaptation to conditions unsuitable for regular crops. Furthermore, their energy content is 10 times greater than that of the best oilseed crops, they can grow all year round, and they require less water than conventional crops, and this can even be brackish. In addition, their growth rate is superior to plants, they fix waste CO_2, and nutrients for their culture can be obtained from wastewater. At the same time, their composition can be modulated by modifying the culture medium, and no pesticides are required (Brennan and Owende, 2010).

Three main types of biomolecules can be accumulated by microalgae: protein, lipids, and starch. Protein can be employed as animal feed, human additives or even as a source for high-value compounds. On the other hand, lipids and starch are mainly used for biofuel production. Lipids can be employed for transesterifications to give biodiesel, whereas starch and other carbohydrates can be hydrolysed into fermentable sugars for bioethanol production (Mata *et al.*, 2010).

Bioethanol from fermentation of sugars derived from starch is one of the most produced biofuels nowadays (Zabed *et al.*, 2017). Starch is the major storage carbohydrate in plants and algae. It is synthesised in the light with the fixed carbon and is degraded in the dark, where photosynthesis is not possible. In terms of structure, starch is formed in insoluble granules composed of two different glucose polymers: amylose and amylopectin. Amylose is a linear chain of glucose units linked by 1α→4

glycosidic bonds, whereas amylopectin is a branched polymer thanks to additional 1α→6 glycosidic bonds between some glucose residues.

Nevertheless, although starch is entirely composed of glucose units, these have to be released so that microorganisms are able to ferment them into ethanol. A possibility to break down starch is based on acid hydrolysis. Despite being a cost-effective method, it entails the generation of big amounts of acid waste, which poses a serious risk for the environment. A more ecological approach to degrade starch into fermentable sugars lies on enzymatic catalysis. Enzymes that are able to hydrolyse starch are widespread among organisms given the ubiquitous presence of this substrate, and these enzymes are generally referred as amylases.

Several types of amylases with different catalytic activities are known. Among them, α-amylases and amyloglucosidases are remarkable in industry. α-amylase has endo-catalytic activity to break 1α→4 glycosidic bonds between glucose units, whilst amyloglucosidase is able to break exocatalytically both 1α→4 and 1α→6 glycosidic bonds that link glucose monomers. Their simultaneous use has widely been studied due to their synergistic behaviour: α-amylase breaks down starch into olygomers that are then readily degraded by amyloglucosidase, which benefits from a higher amount of end-points from which to start hydrolysis.

Although enzymatic degradation of starch is an environmentally-friendly strategy, it entails the critic drawback of high associated costs. During the last decades, several approaches have been proposed to reduce this high price, among which enzyme immobilisation has gained great recognition. Enzyme immobilisation is based on insolubility of the biological catalyst, which enables its recovery from the reaction mixture for reuse. On the contrary, soluble enzyme is lost mixed with the products after its action (Sheldon and van Pelt, 2013).

Many procedures for enzyme immobilisation have already been analysed, such as adsorption to a surface, covalent binding, entrapment, or microencapsulation. Methodologies for covalent binding of the enzyme include not only chemical bonds with a surface, but also cross-linking of enzymes with each other to form Cross-Linked Enzyme Aggregates (CLEAs).

CLEAs overcome the difficulties of soluble enzymes to be recovered after reaction: CLEAs are insoluble aggregates, so they can easily be separated from the solution containing the product. Approaches to retain CLEAs include filtration or centrifugation (Sheldon, 2011). Still, these procedures are far from being perfect: the solution containing the cross-linked enzymes often incorporates other solid residues, and these can attach to the enzymatic aggregates and hinder their recovery. Moreover, enzymes can suffer from compression during these procedures (Sheldon, 2019).

One strategy to bypass this issue relies on the use of superparamagnetic nanoparticles to synthesise magnetic CLEAs (mCLEAs). In this way, the biocatalyst is cross-linked to other enzymes as well as to superparamagnetic nanoparticles. Thus, recovery of mCLEAs can be easily achieved by applying a magnetic field with a magnet. This enables removal of just the product and not the enzymes.

Nevertheless, enzyme immobilisation poses new challenges, since many of the catalytic features of the soluble enzyme are altered. Among the positive aspects, immobilised enzymes benefit from a higher stability in terms of temperature and organic solvents due to a more rigid conformation. However, many drawbacks arise too. Some of these modifications include a different optimum temperature and pH of activity. Moreover, activity tends to be compromised, as immobilisation often causes that the active site of the enzyme is either altered or wrongly oriented, so the substrate is unable to bind. In addition, the mere action of immobilising the enzyme hinders mass transfer and provokes problems for the substrate to diffuse towards the location of the biocatalyst.

Other works have already been done with mCLEAs of amylases, for instance, those of Nadar *et al.* (2016). Still, all those had just used model substrates as maltodextrin or pure starch, but not potential industrial substrates, microalgal extracts.

Previous research in our laboratory had started characterisation of the hydrolysis of microalgal carbohydrates of *Chlorella vulgaris* by commercially available α-amylase and amyloglucosidase, both as soluble enzymes and as mCLEAs. The aim of this work was to continue on that line and gain a better understanding of the alteration of their kinetics as mCLEAs, which will enable optimisation of industrial hydrolysis of starch.

To that end, the effect of a thermal pretreatment of the substrate was studied. This procedure had been found to increase considerably enzymatic activity in previous studies (Souilah *et al.*, 2015).

Several reports also suggested that increasing Ca^{2+} concentration would improve the activity of α-amylase (Dojnov *et al.*, 2008). This enzyme contains four calcium ions, two of which have a fundamental structural role, whereas the two others act as non-essential activators. Hence, the impact of the addition of Ca^{2+} into the microalgal extract for degradation by α-amylase was also analysed.

In terms of mCLEAs, this research aimed to initiate their characterisation for hydrolysis of microalgal carbohydrates, for which the optimum pH of activity was first studied. In addition, the significance of the thermal pretreatment of the substrate was also evaluated.

2. GOALS

This work aimed to continue the characterisation of α-amylase and amyloglucosidase as soluble enzymes and as mCLEAs to hydrolyse microalgal extracts. Specifically, the following points were assessed:

- Effect of a thermal pretreatment of the microalgal extract to improve hydrolysis.
- Effect of Ca^{2+} on the activity of α-amylase.
- Optimum pH of activity of mCLEAs to hydrolyse microalgal extracts.

3. MATERIALS AND METHODS

3.1. ENZYME PREPARATIONS

Commercially available preparations of α-amylase from *Aspergillus oryzae* (A8220, Sigma-Aldrich) and amyloglucosidase from *Aspergillus niger* (A7095, Sigma-Aldrich) were used both for reactions with soluble enzymes and to synthesise mCLEAs.

3.2. MICROALGAL CULTURES

Chlorella vulgaris cells were cultured in modified CHU13 medium (**Supplementary Table 1**) at 25 °C with 150-rpm orbital shaking and continuously illuminated by cool white fluorescent lamps at an intensity of 60 µmol/m^2·s (Chu, 1942). *C. vulgaris* cells were a kind gift from Dr. Sonia Castañón, Neiker, Vitoria-Gasteiz.

Cells were harvested after substantial growth (A_{680} > 1 as a measure of turbidity) and before the death phase, assessed measuring their pigment ratio (carotenoids/chlorophyll) as per the Margalef Index (M.I.), shown in the following equation (Margalef, 1964).

$$\text{M. I.} = \frac{A_{430}}{A_{680}} \quad (1)$$

Briefly, 100 µL of microalgal culture were washed with water in a final volume of 1 mL. Cells were pelleted at 13,000 rpm for 10 min. Then, the supernatant was resuspended in 1.5 mL of methanol. Pigments were extracted incubating the sample at 80ºC for 15 min. Then, the mixture was centrifuged at 13,000 rpm for 10 min, and A_{430} and A_{665} were measured. Thus, only cells with an M.I. of 1-1.5 were harvested.

In order to harvest, cells were pelleted at 9,000 rpm for 5 min, then washed in the reaction buffer (detailed below) with a volume at least higher to the final concentrated volume. Cells were repelleted at 7,000 rpm for 10 min and resuspended in reaction buffer at a concentration of 20 mg of dry microalgae per mL. Microalgal density was assessed by a calibration line plotted with the dry weight of several known volumes for each culture. Cells were lysed with a French press in 5 cycles at 1,250 psi and stored at -20ºC until use.

3.3. SYNTHESIS OF mCLEAS

Samples of 25 µL of α-amylase or 35 µL of amyloglucosidase in a final aqueous volume of 4.5 mL were mixed with 30 mg of ferric oxide nanoparticles with a mean diameter of 10 nm. These were synthesised as per Cruz-Izquierdo *et al.* (2014) and López *et al.* (2014). These superparamagnetic nanoparticles had been previously functionalised with amino groups on their surface for cross-linking with enzymes.

Enzymes were precipitated by adding 42 mL of 3.6 M ammonium sulphate to the mixture. After 5 min at RT with 30-rpm oscillatory rotation, 3.72 mL of 200 mM glutaraldehyde (the crosslinking agent) were added to the mixture. The cross-linking reaction between enzymes and magnetic nanoparticles was incubated for 24 h at RT with 30-rpm oscillatory rotation.

The resulting mCLEAs were retained with a magnet and washed three times with PBS buffer. The Schiff bases formed during the cross-linking reaction were reduced with 100 mM carbonate-bicarbonate buffer, pH 10, containing 1 mg/mL $NaBH_4$. The reducing reaction was incubated for 2 h at RT with 30-rpm oscillatory rotation. Then, mCLEAs were washed with PBS, containing 2 M NaCl to remove unspecific interactions. Then, mCLEAs were washed three times with PBS, once with PBS, 1% (v/v) Triton X-100, and three times with PBS for a final concentration of 2 mg of mCLEAs per mL. mCLEAs were stored at 4ºC until use.

3.4. REACTIONS

3.4.1. Reactions with soluble enzymes

Reactions for hydrolysis of microalgal starch were carried out with α-amylase and/or amyloglucosidase in 3 mL of microalgal lysate in 50 mM acetate, pH 4.6 buffer at 42.5°C with 35-rpm oscillatory rotation, which research in the laboratory had found as the optimum conditions.

If pure potato starch (Sigma-Aldrich) was used as a substrate instead of microalgal lysate, said starch was mixed in reaction buffer at a concentration of 10 mg/mL. For reactions with a thermal pretreatment (when stated), the substrate (either microalgal lysate or starch) was heated at 90ºC for 5 min in a water bath without the enzyme (which was added after cooling of the substrate). For reactions with Ca^{2+} (when stated), $CaCl_2$ was added to the reaction mixture (either microalgal lysate or starch) prior to a possible thermal pretreatment. For reactions with dialysed enzyme, the enzyme was eluted through Sephadex G-25 in a PD-10 desalting column (GE Healthcare).

To determine hydrolysis in the reaction, the DNS method described by Miller (1959) was followed. Briefly, samples of 375 µL were centrifuged at 13,000 rpm for 10 min. 10 µL of the supernatant were saved for analysis by thin-layer chromatography and

250 µL were mixed with 750 µL of DNS solution (44 mM 3,5-dinitrosalicylic acid, 4 mM Na_2SO_3, 250 mM NaOH). The mixture was heated at 100ºC for 10 min. Then, it was diluted with 4 mL of water and A_{540} was measured. To estimate the concentration of reducing sugars in the reacted sample, a calibration line plotted with known concentrations of D-glucose was used.

The degree of hydrolysis in the reaction over the whole microalgal lysate was estimated determining total sugars in the lysate by the phenol-sulphuric acid method (Dubois *et al.*, 1956): 4 µL of microalgal lysate were mixed with 396 µL of water, 400 µL of 5% (w/v) phenol, and 2 mL of sulphuric acid. The mixture was incubated at RT for 30 min. Then, A_{490} was measured. To estimate total sugars, a calibration line plotted with known concentrations of D-glucose was used.

3.4.2. Reactions with mCLEAs

Reactions for hydrolysis of microalgal starch were carried out with 1 mg of the aforementioned mCLEAs of α-amylase or amyloglucosidase per mL of microalgal lysate (3 or 4 mL in total) at 55°C with 35-rpm oscillatory rotation, which research in the laboratory had found as the optimum conditions. In order to determine the optimum pH of activity, 50 mM acetate, pH 4.6 buffer; 50 mM phosphate, pH 6 buffer; and 50 mM phosphate, pH 8 buffer were used. mCLEAs were washed with reaction buffer three times before use.

When commercially available potato starch was used as a substrate instead of microalgal lysate, said starch was mixed in reaction buffer at a concentration of 10 mg/mL. For reactions with a thermal pretreatment (when stated), the substrate (either microalgal lysate or starch) was heated at 90ºC for 5 min in a water bath without mCLEAs (which were added after cooling of the substrate). For reactions with Ca^{2+} (when stated), $CaCl_2$ was added to the reaction mixture (either microalgal lysate or starch) prior to a possible thermal pretreatment.

To determine the hydrolysis in the reaction, mCLEAs in samples of 500 µL were removed with a magnet (**Supplementary Figure 1**). Then, samples were centrifuged at 13,000 rpm for 10 min, and the same procedure as for samples of reactions with soluble enzymes was followed.

3.5. THIN-LAYER CHROMATOGRAPHY (TLC)

Degradation of substrates and appearance of products in microalgal starch hydrolysis was assessed by thin-layer chromatography (TLC). 10 µL of saved supernatant of the collected samples from reactions were applied on TLC Silica gel 60 plates (Merck) that had been previously dried at 100ºC for 30 min. The fingerprint profile was developed in 4 h with a ternary mixture of 2-buthanol, 2-propanol, and water in a ratio of 2.5:1.2:1 (v/v). For visualisation, the sheet was sprayed with a solution containing 30 mM carbazole in ethanol and sulphuric acid in a ratio 19:1 (v/v). Then, the plate was incubated at 100ºC for 10 min.

4. RESULTS AND DISCUSSION

4.1. SOLUBLE ENZYME

4.1.1. Thermal pretreatment

Starch is the major storage carbohydrate in *C. vulgaris*. However, starch forms compact, insoluble granules that are difficult to be accessed by enzymes. Thus, heating starch has proved to be an effective approach to loosen this rigid structure and enable glycosidases to break it down into olygo- or monosaccharides. Souilah *et al.* (2015) described a positive effect on the degradation of carbohydrates by amylases. In order to test that potentially positive impact, a thermal pretreatment was applied to the microalgal lysate by heating it at 90ºC for 5 minutes prior to the addition of α-amylase and/or amyloglucosidase.

4.1.1.1. α-amylase

Degradation of carbohydrates in the pretreated microalgal lysate was remarkably higher for a broad range of α-amylase concentrations (**Figure 1A**). Furthermore, the substrates were hydrolysed soon after addition of the enzyme into the pretreated lysate, what made enzyme activity plateau rapidly. However, in the case of non-treated microalgal extract, activity was notably lower and progressed linearly.

Interestingly, at higher enzyme concentrations, activity on non-treated lysate reached the same degree of hydrolysis after 60 minutes (**Figure 1B**). Nevertheless, higher

differences in activity between non-treated and pretreated extract were noticed at lower enzyme concentrations. This phenomenon was especially significant at the minimum assayed concentration (0.0045 µL of α-amylase per mg of microalgal lysate), where non-treated substrate was barely degraded. Moreover, this enzyme concentration of 0.0045 µL/mg on pretreated lysate reached the same degree of hydrolysis than a 10-fold higher concentration of enzyme on non-treated substrate, and the degradation was even more rapid.

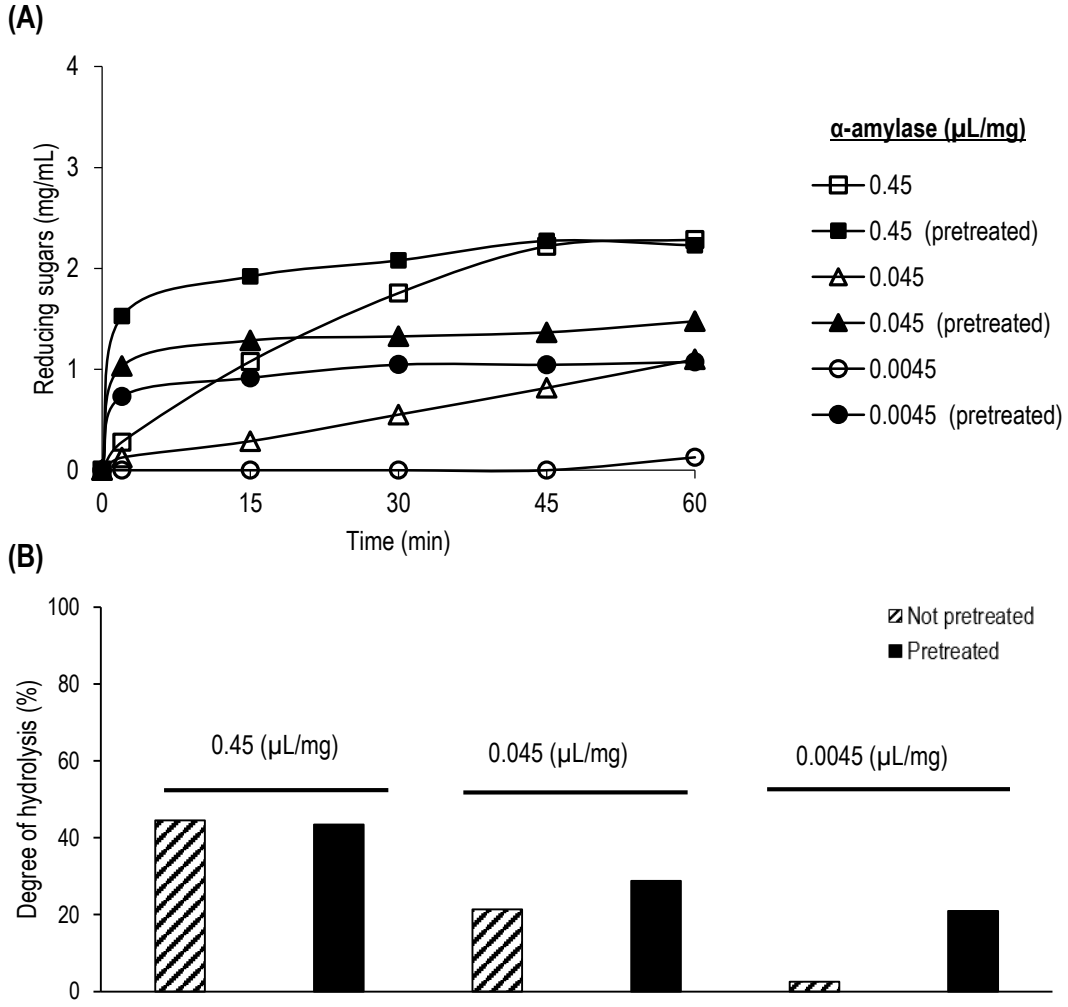

Figure 1. Effect of the thermal pretreatment of microalgal lysate on the carbohydrate hydrolysis catalysed by α-amylase. A) Progress of the carbohydrate hydrolysis. B) Degree of hydrolysis after 60 min. The pretreated lysate was more susceptible to enzymatic degradation at all assayed concentrations of α-amylase. Microalgal lysate (20 mg of dry microalgae / mL) was heated at 90ºC for 5 min before addition of the enzyme. Reactions were carried out at 42.5ºC in 50 mM acetate, pH 4.6 buffer. The axis of ordinates in A) shows values of reducing sugars up to 4 mg/mL to enable a better comparison with the activity by amyloglucosidase (**Figure 2A**) and α-amylase and amyloglucosidase combined (**Figure 3A**).

However, the degree of hydrolysis was estimated following degradation of all glycosidic bonds in the extract by phenol-sulphuric acid degradation. α-amylase, instead, breaks only 1α→4 glycosidic bonds between glucose residues. Therefore, the real hydrolysis by this enzyme over the total amount of bonds which is able to break must be greater than the percentage shown.

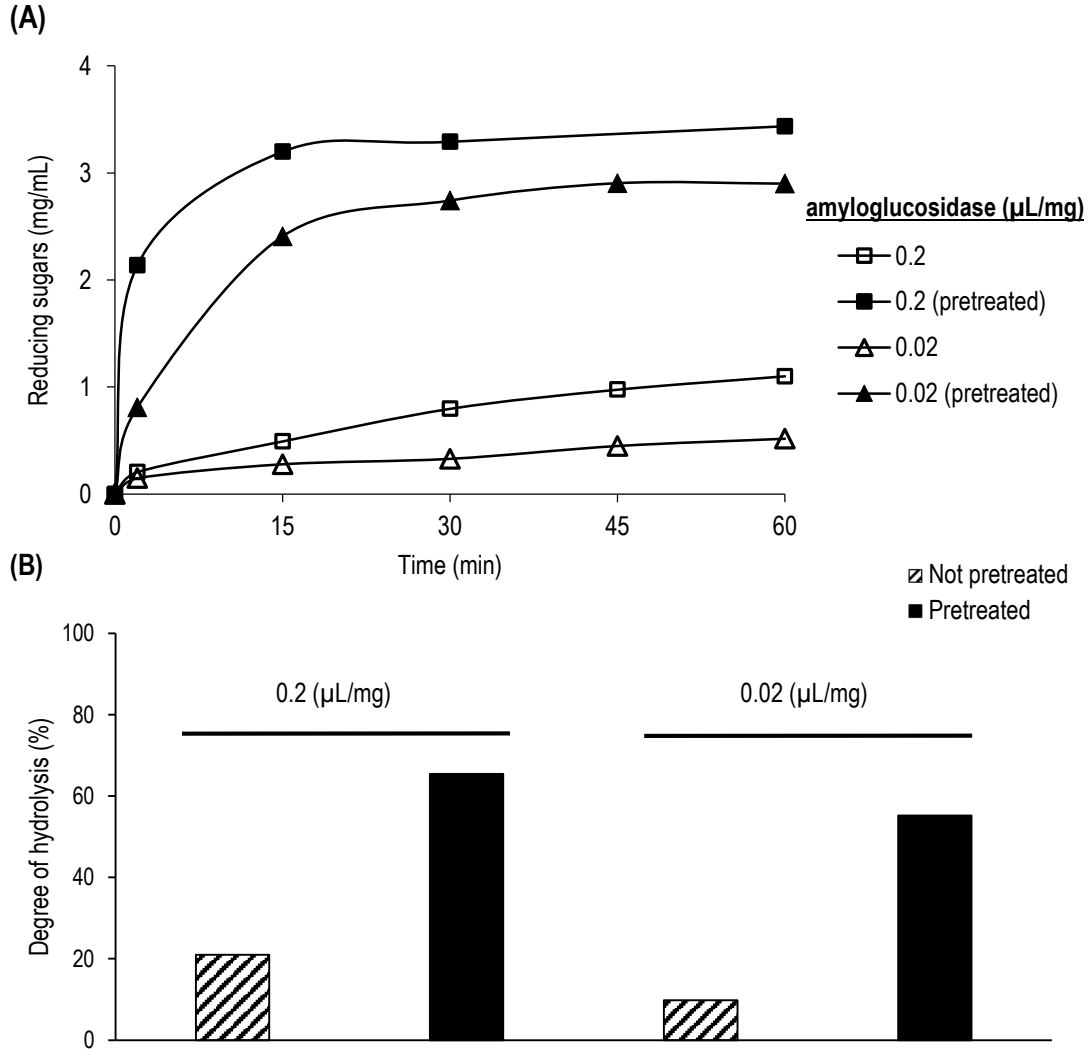

Figure 2. Effect of the thermal pretreatment of microalgal lysate on the carbohydrate hydrolysis catalysed by amyloglucosidase. A) Progress of the carbohydrate hydrolysis. B) Degree of hydrolysis after 60 min. The pretreated lysate was more susceptible to enzymatic degradation at all assayed concentrations of amyloglucosidase. Microalgal lysate (20 mg of dry microalgae / mL) was heated at 90ºC for 5 min before addition of the enzyme. Reactions were carried out at 42.5ºC in 50 mM acetate, pH 4.6 buffer. Concentrations are shown as volume of added enzyme per mass of microalgal lysate.

4.1.1.2. Amyloglucosidase

In the case of amyloglucosidase, the positive impact of a thermal pretreatment was even more remarkable (**Figure 2A**). Thus, the pretreated extract a 3-fold greater yield of reducing sugars than the non-treated substrate. In a similar way to α-amylase, enzymatic degradation begins rapidly and plateaus after few minutes for the pretreated lysate, wheares it advances slowly and linearly for the non-treated extract.

Noticeably, the degree of hydrolysis on the pretreated lysate for enzyme concentrations of 0.2 and 0.02 µL/mg after 60 minutes was similar, despite the concentration being 10-fold lower (**Figure 2B**). As in the case of α-amylase, the degree of hydrolysis showed a value lower than the real one due to the methodological limitation. Still, this value was greater for amyloglucosidase, since it is able to break both 1α→4 and 1α→6 glycosidic bonds between glucose residues.

Overall, the thermal loosening of starch granules showed a more significant effect for amyloglucosidase than for α-amylase. The latter is an enzyme with endocatalytic activity, and, therefore, it is able to break down long glucose polymers with 1α→4 glycosidic bonds at any position of the chain. Nevertheless, although amyloglucosidase is also able to debranch starch by removing 1α→6 glycosidic bonds apart from the 1α→4 glycosidic bonds, it shows an exocatalytic activity. Consequently, it requires binding to the end of a polymer prior to starting degradation. Hence, loosening of starch could enable amyloglucosidase to access more end-points from which it could begin degradation. This would explain the observed higher increase in activity for amyloglucosidase over α-amylase.

4.1.1.3. α-amylase and amyloglucosidase combined

To further characterise the effect of heating the substrate before the enzymatic treatment, the concomitant activity of α-amylase and amyloglucosidase was analysed. In this case, the intermediate concentration used for α-amylase alone was employed, i.e, 0.045 µL of enzyme per mg of microalgal extract. In the case of amyloglucosidase, the same concentration as α-amylase or a 4-fold higher concentration was used.

Both assayed concentrations showed again a notable increase in activity with the pretreated substrate (**Figure 3A**). Thus the positive effect of the thermal treatment was

confirmed. As in the assays with the enzymes individually, pretreated lysate quickly yielded a big amount of reducing sugars and then plateaud, whereas degradation of non-treated substrate increased slowly and in a linear fashion.

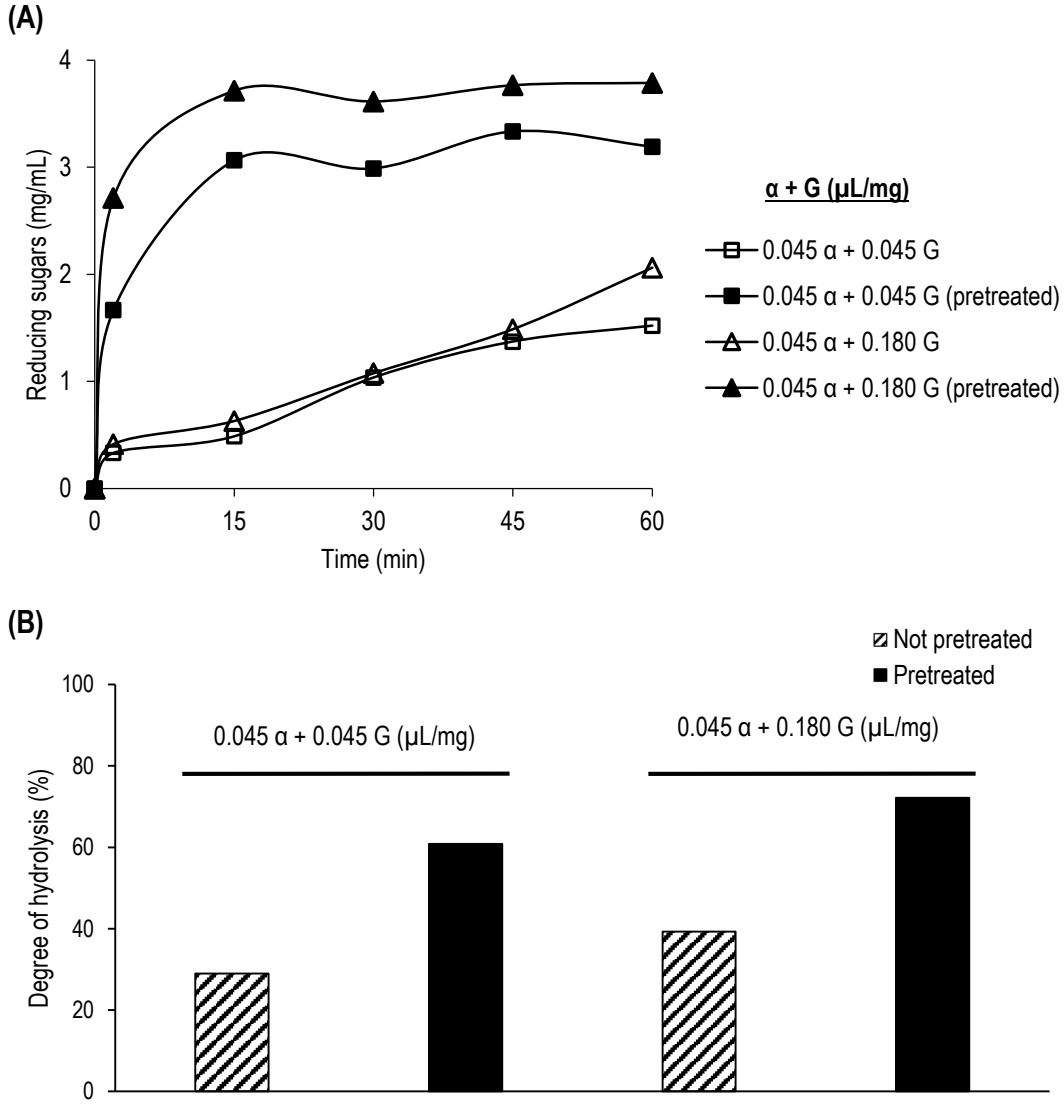

Figure 3. Effect of the thermal pretreatment of microalgal lysate on the carbohydrate hydrolysis catalysed by α-amylase and amyloglucosidase combined. A) Progress of carbohydrate hydrolysis. B) Degree of hydrolysis after 60 min. The pretreated lysate was more susceptible to enzymatic degradation at all assayed concentrations of α-amylase and amyloglucosidase. Microalgal lysate (20 mg of dry microalgae / mL) was heated at 90ºC for 5 min before addition of the enzymes. Reactions were carried out at 42.5ºC in 50 mM acetate, pH 4.6 buffer. Concentrations are shown as volume of added enzyme per mass of microalgal lysate. *α* refers to α-amylase, and *G*, to amyloglucosidase.

However, a big rise in the concentration of amyloglucosidase did not show such a great increment in the carbohydrate degradation, either for pretreated or for non-treated microalgal extract. This phenomenon was also observed at lower enzyme concentrations (**Supplementary Figure 2**). Consequently, the degree of hydrolysis after 60 minutes was comparable for the two different ratios of enzymes, both with and without the thermal treatment of the substrate (**Figure 3B**). Still, analysis by TLC showed how hydrolysis with both enzymes combined progressed differently to each of the enzymes individually (**Supplementary Figure 3**).

4.1.2. Non-essential activation of α-amylase by Ca^{2+}

α-amylase contains four Ca^{2+}, two of which play a structural role. The two other Ca^{2+}, instead, promote enzymatic catalysis, but are not essential for activity (Dojnov *et al.*, 2008). In order to study the significance of this phenomenon, the addition of Ca^{2+} at concentrations of 2, 5, and 8 mM into microalgal lysate was assessed.

The presence of Ca^{2+} at all assayed concentrations did increase the enzymatic activity (**Figure 4A**). Nonetheless, all 2, 5, and 8 mM concentrations showed a very similar effect, and the rise in activity over the enzyme without Ca^{2+} was insignificant and not comparable to other data found by Dojnov *et al.* (2008), who also found inhibition at high concentrations of Ca^{2+}. Still, said work assayed a broader range of concentrations (0-100 mM) and employed a different α-amylase.

In any case, it was hypothesised that either the microalgal extract could already contain enough Ca^{2+} as to enhance enzymatic activity, since the impure culture medium contained Ca^{2+} that can be absorbed by the cells. Another possibility was that the commercial enzyme itself contained Ca^{2+} as a preservative to maintain stability.

To further analyse these hypotheses, dialysed α-amylase with and without addition of Ca^{2+} was assayed to degrade potato starch. Thus, dialysis showed a negative effect on the enzymatic activity, denoting that the commercial α-amylase already contained Ca^{2+} (**Figure 4B**). Still, the addition of Ca^{2+} further increased the degradation of starch in both dialysed and non-dialysed α-amylase, and this happened in a more significant way than when microalgal extract was assessed. Therefore, Ca^{2+} in the microalgal lysate would already be promoting considerably the activity of the assayed α-amylase.

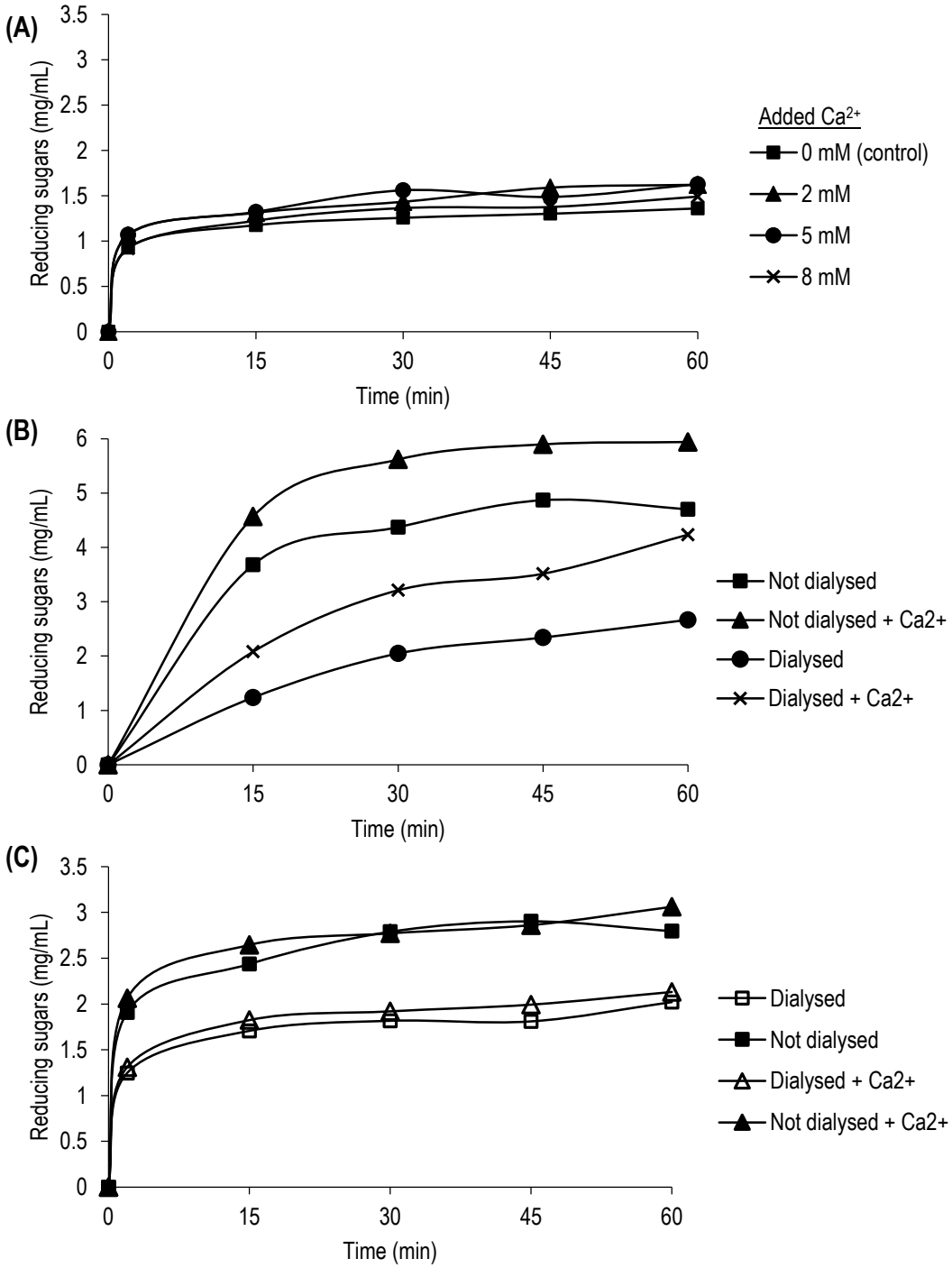

Figure 4. Effect of Ca^{2+} on the carbohydrate hydrolysis catalysed by α-amylase. A) Progress of carbohydrate hydrolysis of microalgal lysate with Ca^{2+}. The presence of Ca^{2+} promoted slightly enzymatic activity. Microalgal lysate was heated at 90ºC for 5 min before addition of the enzyme (0.0045 µL of α-amylase / mg of microalgal lysate). B) Progress of starch hydrolysis with and without Ca^{2+} with dialysed and non-dialysed α-amylase. Dialysis had a negative impact on activity, whereas the presence of Ca^{2+} posed a positive effect. Starch was heated at 90ºC for 5 min before addition of the enzyme (0.03 µL of α-amylase / mg of starch). Ca^{2+} was added at a concentration of 2 mM. C) Progress of carbohydrate hydrolysis of dialysed and non-dialysed microalgal lysate with and without Ca^{2+}. Dialysis decreased activity, whereas addition of Ca^{2+} barely had any positive effect. Microalgal lysate was heated at 90ºC for 5 min before addition of the enzyme (0.045 µL of α-amylase / mg of microalgal lysate). Ca^{2+} was added at a concentration of 2 mM. All reactions were carried out at 42.5ºC in 50 mM acetate, pH 4.6 buffer. The axis of ordinates in A) shows values of reducing sugars up to 3.5 mg/mL to enable a better comparison with the activity with dialysed microalgal extract in C).

In order to ascertain such results, the hydrolysis of dialysed and non-dialysed microalgal lysate with and without additional Ca^{2+} was also assessed. This experiment showed that the dialysed substrate yielded remarkably less product, which confirms the importance of the Ca^{2+} present in the extract to promote the catalysis by α-amylase (**Figure 4C**).

Furthermore, the addition of external Ca^{2+} at a concentration of 2 mM barely increased the enzymatic activity, unlike when commercial starch was degraded (**Figure 4B**). This suggests that the concentration of Ca^{2+} in the microalgal extract must be considerably higher. At the same time, these data imply that *C. vulgaris* cells must accumulate a significant amount of Ca^{2+}, since their internal concentration must be remarkably greater than 2 mM, but the initial concentration of the culture medium was 0.73 mM (**Supplementary Table 1**).

4.2. mCLEAs

4.2.1. Optimum pH of activity

One of the initial steps to characterise carbohydrate degradation in microalgal lysate with mCLEAs was to search for the optimum pH of activity, as enzyme immobilisation often provokes variations in comparison with the soluble biocatalyst. Thus, three different pH values were assayed: 4.6, 6, and 8.

However, the spectrophotometric methodology employing DNS was unsuccessful to unveil the enzymatic activity at pH 6 and 8 for mCLEAs of α-amylase and amyloglucosidase. At those pH values, extracted pigments remained in the soluble fraction after the reaction, which caused interference when the absorbance was read (data not shown). In order to overcome said methodological difficulty, the progress of the reaction was semiquantitatively analysed by TLC.

4.2.1.1. mCLEAs of α-amylase

In the case of mCLEAs of α-amylase, pH 4.6 showed the greatest progress in carbohydrate hydrolysis among the assayed pH values (**Figure 5A**). At pH 6, instead, the spot corresponding to hydrolysed carbohydrates barely increased in size or intensity over time and was fainter. At pH 8, these phenomena associated with the lack

of activity were even more remarkable. Still, it was notable that, even at pH 4.6, the spot of hydrolysed carbohydrates did not enlarge very notably since the initial time, what could denote a very low activity. This might well be a consequence of enzyme immobilisation, which tends to increase stability but decrease activity.

4.2.1.2. mCLEAs of amyloglucosidase

Also like for mCLEAs of α-amylase, the optimum pH of activity proved to be 4.6 (**Figure 5B**). At this pH, the spot of hydrolysed carbohydrates developed the greatest brightness and size, whereas activity decreased at pH 6, and even more at pH 8. However, in the same way as for mCLEAs of α-amylase, even at this optimum pH, enzymatic activity seemed to be low: the spot of hydrolysed carbohydrates was already considerable at the initial time and did not show a great development.

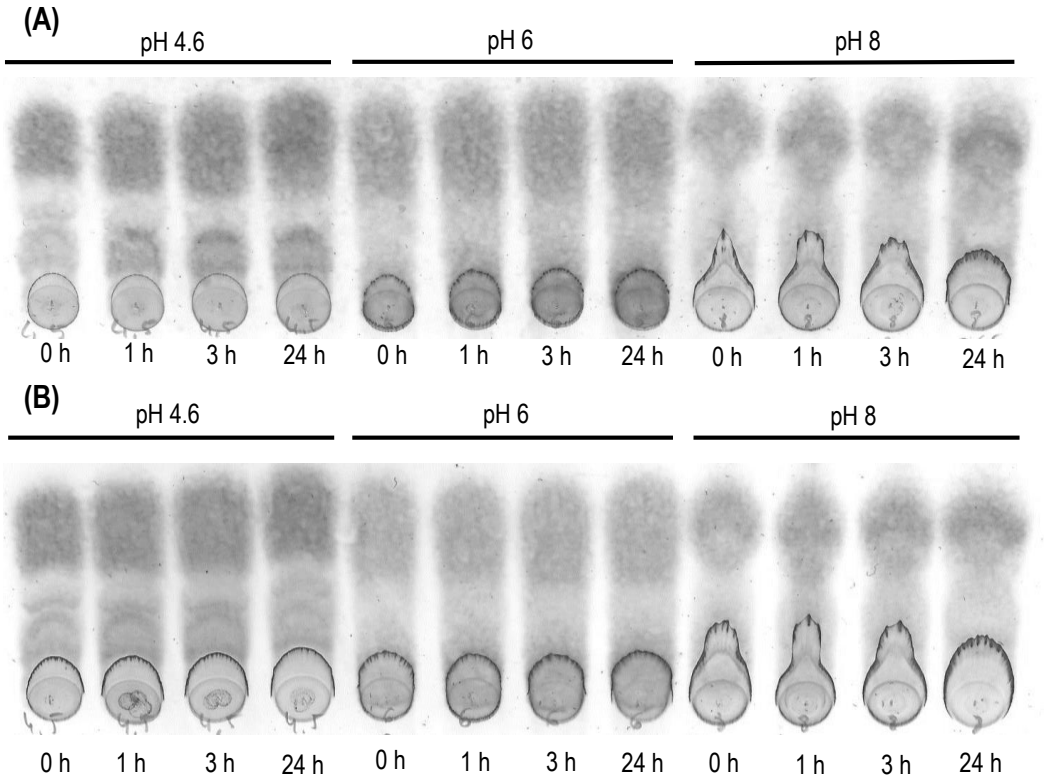

Figure 5. Optimum pH of activity of mCLEAs. A) Analysis by TLC of the progress of carbohydrate hydrolysis by mCLEAs of α-amylase. B) Analysis by TLC of the progress of carbohydrate hydrolysis by mCLEAs of amyloglucosidase. The spot corresponding to pH 4.6 showed the most significant increase in size and brightness over time among the assessed pH values for both types of mCLEAs. Reactions were carried out with 1.33 mg of mCLEAs / mL of microalgal lysate (20 mg of dry microalgae / mL) at 55ºC in 50 mM acetate, pH 4.6; 50 mM phosphate, pH 6; or 50 mM phosphate, pH 8 buffers.

4.2.2. Thermal pretreatment

To test whether thermal loosening of starch in the microalgal lysate could promote activity also in mCLEAs, pretreatment of the substrate was analysed as with the soluble enzymes.

4.2.2.1. mCLEAs of α-amylase

When mCLEAs of α-amylase were used to degrade carbohydrates in microalgal extract at the optimum pH, no activity was found either with pretreated or non-treated substrate, with or without additional Ca^{2+} (**Figure 6A**). Several batches of synthesised mCLEAs were tested, so the possibility that it could be a problem from a single batch was discarded. This questioned if the supposedly optimum pH which was previously found was right, since, even with TLC, activity seemed to be very low (**Figure 5A**).

In order to check whether at pH 6 the mCLEAs would have any activity, more experiments were conducted in these conditions. The aforementioned problem of solubility of microalgal pigments proved to disappear with pretreated samples after 24 h of reaction, what allowed the activity to be read spectrophotometrically. Still, no activity was found after 24 h of reaction (**Figure 6B**).

The possibility that suspended residues of the microalgal extract after lysis could attach to the mCLEAs was also considered. Thus, residues could impede contact between enzymes and substrates. Immobilised enzymes suffer from a more rigid position, and the mCLEAs themselves aggregated with each other. Therefore, suspended residues of the lysate also adsorbed, and this additional steric hindrance might abolish entirely the entrance of the substrate to the catalytic centre of α-amylase.

Aiming to test this hypothesis, mCLEAs of α-amylase were incubated with potato starch at pH 4.6, which was meant to be the optimum for activity. Nevertheless, no hydrolysis occurred although there were no suspended residues (**Figure 6C**).

Considering these data altogether, this lack of activity should not be a problem of a single batch of mCLEAs of α-amylase nor of the microalgal lysate. It could be that, in the case of this enzyme, the proportion of magnetic nanoparticles and enzyme is not appropriate and that most of the enzymes lye trapped surrounded by nanoparticles, unable to receive any substrate.

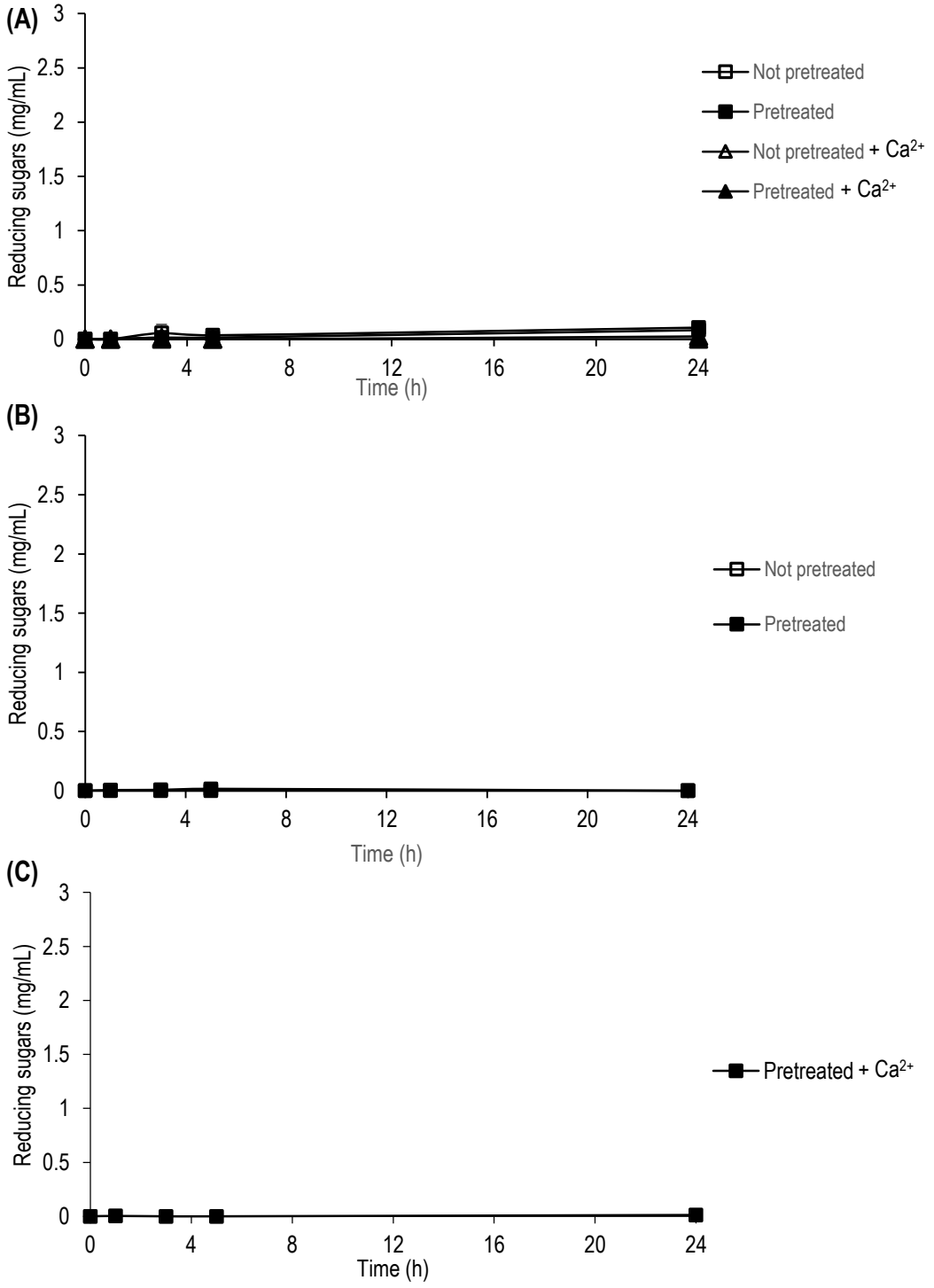

Figure 6. Effect of the thermal pretreatment on the carbohydrate hydrolysis catalysed by mCLEAs of α-amylase. A) Progress of the carbohydrate hydrolysis of microalgal lysate at pH 4.6. No activity was found with or without pretreatment and with or without additional Ca^{2+}. B) Progress of the carbohydrate hydrolysis of microalgal lysate at pH 6. No activity was found with or without pretreatment. C) Progress of starch hydrolysis at pH 4.6. No activity was found with pretreatment and Ca^{2+}. The substrate was heated at 90ºC for 5 min before addition of the mCLEAs. Ca^{2+} was added at a concentration of 2 mM. Reactions were carried out with 1 mg of mCLEAs / mL of microalgal lysate (20 mg of dry microalgae / mL) or starch (10 mg/mL) at 55ºC in 50 mM acetate, pH 4.6; or 50 mM phosphate, pH 6 buffers. Values are the mean ± the standard error mean of two to six replicate reactions. The axis of ordinates shows values of reducing sugars up to 3 mg/mL to enable a better comparison with the activity of mCLEAs of amyloglucosidase (**Figure 7**).

Another possibility is that the followed procedure for immobilisation somehow denatured α-amylase, and, therefore, no activity was found. Here, the enzyme precipitated by addition of ammonium sulphate. Other works (Talekar *et al.*, 2012; Nadar *et al.*, 2016) also assayed with *n*-propanol, acetone, and DMSO, among others, which could be an alternative to assay.

It could also be that, for this enzyme, the cross-linker (glutaraldehyde here) is too short to enable the right position of α-amylase to catalyse hydrolysis of carbohydrates. Tests with a longer cross-linking arm could also elucidate more about this hypothesis. For instance, Nadar *et al.*, (2016) overcame loss of activity in α-amylase with macromolecular cross-linkers, including agar, chitosan, dextran, and gum arabic.

Additionally, the data also suggest that a further confirmation of the optimum pH of activity of mCLEAs of α-amylase should be required.

4.2.2.2. mCLEAs of amyloglucosidase

In the case of mCLEAs of amyloglucosidase, immobilised enzymes were indeed able to degrade microalgal carbohydrates in pretreated substrate at pH 4.6 (**Figure 7**). Here, the starch whose structure had been loosen was readily hydrolysed by mCLEAs, whereas untreated extract was barely degraded. This also suggests that another test to confirm its optimum pH of activity would be reasonable: previous assays to confirm the optimum pH of activity were accomplished with non-treated extract, where activity was negligible.

Interestingly, the effect of the thermal pretreatment showed even a greater impact on catalysis in mCLEAs than in the soluble enzyme. In this way, soluble amyloglucosidase hydrolysed treated carbohydrates very quickly and then plateaued (**Figure 2A**). mCLEAS, instead, showed a rather linear progress of degradation at the beginning.

It is also noticeable how immobilisation of the enzyme in mCLEAs turned catalysis remarkably slower: 24 hours were required to achieve the same yield of reducing sugars as what soluble amyloglusidase reached in around 15 minutes. Thus, only about 1% of the activity was retained. However, other works, amyloglusidase retained 92.8%

(Gupta *et al.*, 2013) and 85.3% (Nadar and Rathod, 2016) of activity after immobilisation as mCLEAs, also with glutaraldehyde as the cross-linker. With pectin as the cross-linking agent, Nadar and Rathod (2016) even increased the recovered activity to 95.4%. Still, such results were obtained employing pure starch (Gupta *et al.* 2013) or maltodextrin (Nadar and Rathod, 2016) as substrates. As discussed for mCLEAs of α-amylase, microalgal extract contained numerous suspended particles from lysis that attached to mCLEAs and that might prevent contact between substrate and immobilised enzyme.

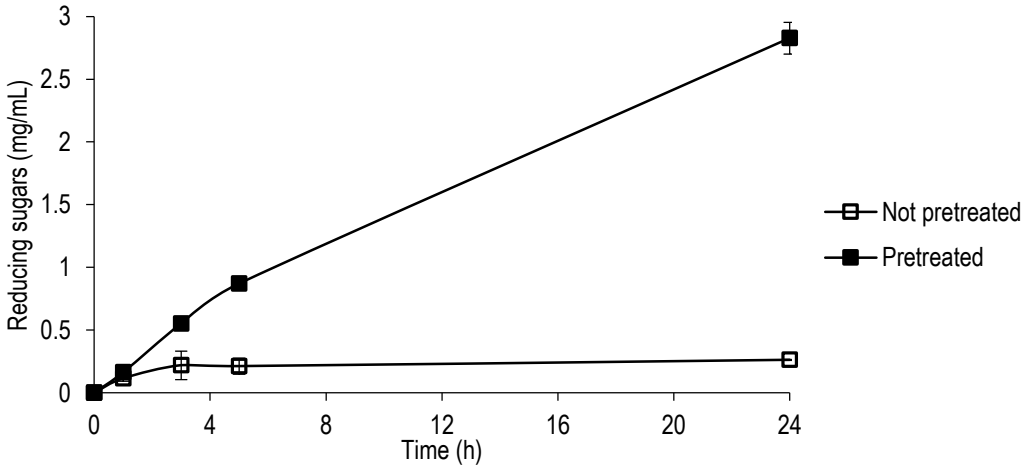

Figure 7. Effect of the thermal pretreatment on the carbohydrate hydrolysis catalysed by mCLEAs of amyloglucosidase. Progress of carbohydrate hydrolysis of microalgal lysate at pH 4.6. Pretreated lysate was more susceptible to enzymatic degradation. Microalgal lysate was heated at 90ºC for 5 min before addition of the mCLEAs. Reactions were carried out with 1 mg of mCLEAs / mL of microalgal lysate (20 mg of dry microalgae / mL) at 55ºC in 50 mM acetate, pH 4.6 buffer. Values are the mean ± the standard error mean of two replicate reactions.

5. MAIN CONCLUSIONS AND FUTURE PERSPECTIVES

This work shows the importance of the thermal pretreatment of microalgal starch to improve the hydrolytic activity, and, therefore, to increase the yield of sugars, by α-amylase and amyloglucosidase, both individually and combined, In the case of soluble enzymes, loosening of the compact structure of starch by a high temperature showed a very quick degradation of its polymers. On the other hand, hydrolysis of untreated

substrate was slowed down at high enzyme concentrations, whilst negligible activity was detected in the presence of little enzyme.

As for mCLEAs, the optimum pH of activity for both immobilised α-amylase and amyloglucosidase was pH 4.6, among the different tested pH values. Still, a further analysis is required to ascertain such results. With regard to the thermal pretreatment, immobilised amyloglucosidase showed negligible capacity to degrade untreated microalgal carbohydrates. Nevertheless, heating of the substrate did enable hydrolysis, although immobilisation of the enzyme decreased its activity considerably.

A further analysis is necessary in the case of mCLEAs of α-amylase, which were unable to break down treated or untreated carbohydrates either from microalgal lysate or commercial starch. A more comprehensive study of the procedure for the synthesis of these mCLEAs might elucidate the reason for their lack of activity. Test with other precipitants and longer cross-linking arms could help solve this issue.

Also, this research proves the remarkable increase in α-amylase activity by Ca^{2+}. Albeit, external addition of Ca^{2+} into the reaction mixture for degradation of microalgal carbohydrates did not enhance considerably the yield of released sugars. The reasons for this phenomenon were that the commercial preparation of the enzyme already contained Ca^{2+} and, most notably, that Ca^{2+} abounded in the microalgal extract itself.

Nevertheless, there is still work to be completed. Apart from fixing the issue that deters mCLEAs of α-amylase from showing any activity, other aspects of the synthesis of mCLEAs should also be double checked. This could also help mCLEAs of amyloglucosidase retain more activity to achieve the high values of recovery reported in literature (Gupta *et al.*, 2013; Nadar and Rathod, 2016).

Furthermore, the hydrolytic process of mCLEAs of α-amylase and mCLEAs of amyloglucosidase combined should be studied to ascertain which proportions fit best. Moreover, synthesis of combi-mCLEAs with both cross-linked α-amylase and amyloglucosidase could also be an idea to analyse, as it already proved successful, even with pullulanase as a third amylase (Talekar *et al.*, 2013; Talekar *et al.*, 2017). Besides, this mCLEAs might help α-amylase retain activity after immobilisation,

which could be tested by comparing the activity of these mCLEAs with mCLEAs of amyloglucosidase alone in the same concentration.

Finally, after achieving a high retention of both enzymatic activities in the mCLEAs, reuse of these immobilised biocatalysts should be investigated. This would reveal the amount of hydrolytic cycles for which the mCLEAs could be employed, which is one of the main goals of immobilised enzymes. Consequently, a cost-effective, ecological method competitive with acid hydrolysis could be attained to degrade starch industrially.

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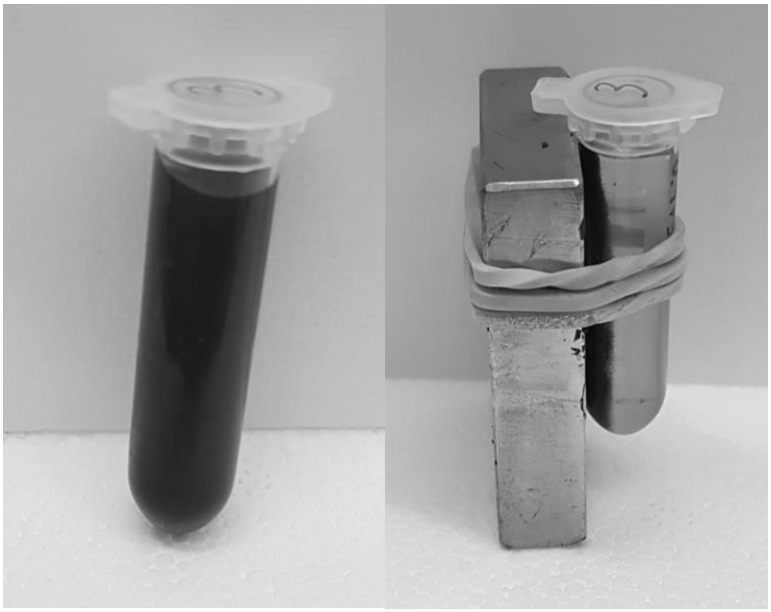
ANNEXE: SUPPLEMENTARY INFORMATION

SUPPLEMENTARY TABLES

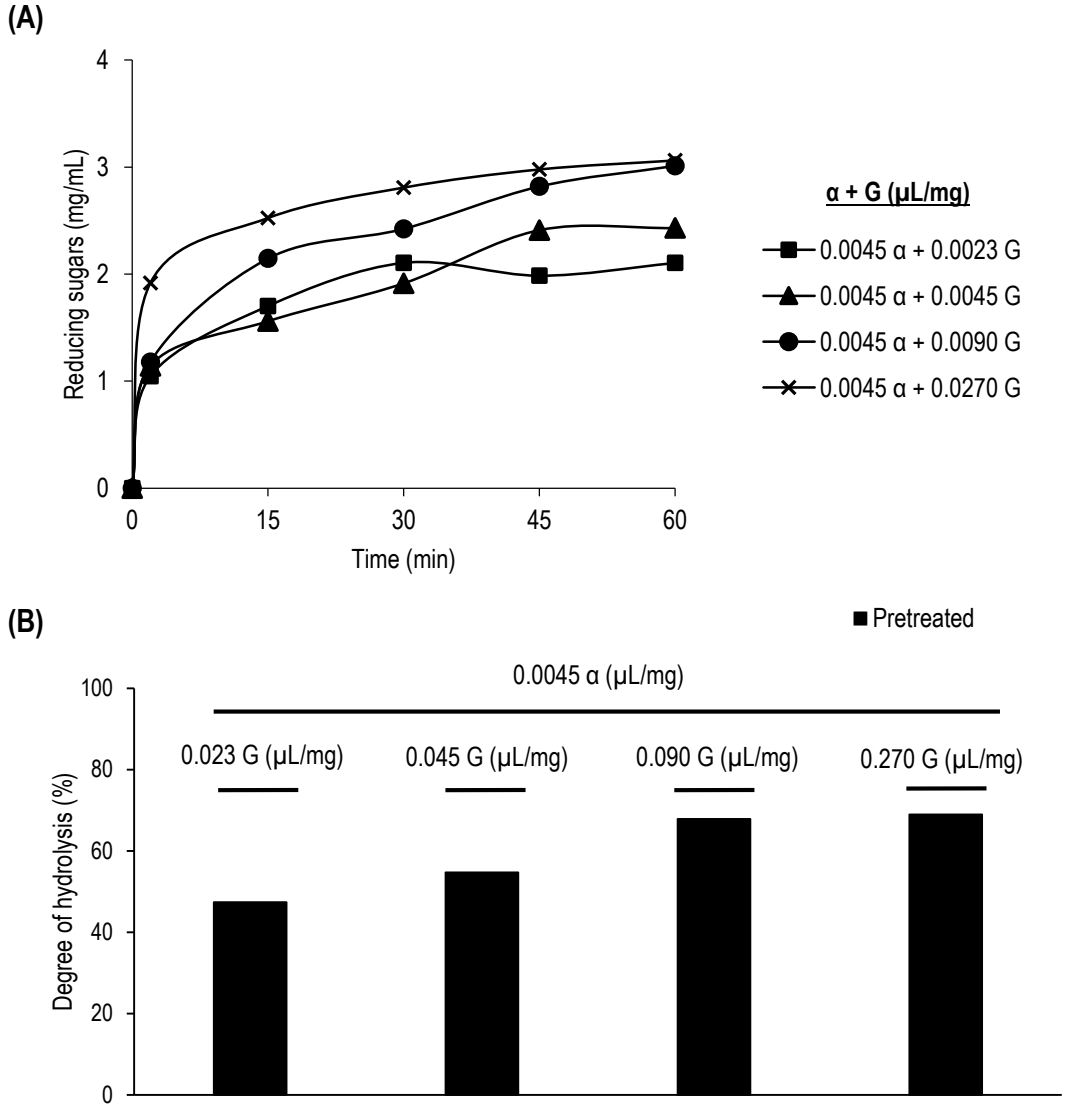
Supplementary Table 1. Composition of the modified CHU13 medium. pH was adjusted to 7.5 with HCl and KOH.

Component	Concentration (µM)
KNO3	3950
MgSO4	810
CaCl2	730
Citric acid	520
K2HPO4	460
H3BO3	92
Ferric citrate	81
MnCl2	18
ZnSO4	1.5
CuSO4	0.6
Na2MoO4	0.4
CoCl2	0.2

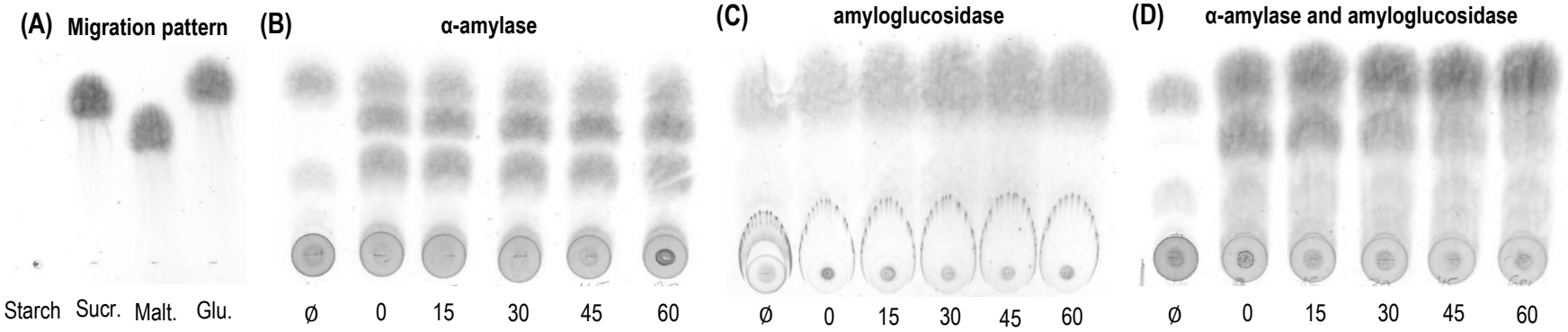
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Separation of mCLEAs by a magnetic field. mCLEAs can be readily recovered by a magnet from a reaction mixture for their reuse. Left: freely suspended mCLEAs. Right: mCLEAs separated from the bulk solution by a magnet.



Supplementary Figure 2. Effect of the α-amylase:amyloglucosidase ratio on the hydrolysis of carbohydrates of pretreated microalgal extracts catalysed by α-amylase and amyloglucosidase combined. A) Progress of carbohydrate hydrolysis. B) Degree of hydrolysis after 60 min. A considerable increase in the concentration of amyloglucosidase did not correspond with a notably higher hydrolysis. Microalgal lysate (20 mg of dry microalgae / mL) was heated at 90ºC for 5 min before addition of the enzymes. Reactions were carried out at 42.5ºC in 50 mM acetate, pH 4.6 buffer. Concentrations are shown as volume of added enzyme per mass of microalgal lysate. *α* refers to α-amylase, and *G*, to amyloglucosidase.



Supplementary Figure 3. Progress of the microalgal carbohydrate hydrolysis followed by TLC. A) Migration pattern of starch, sucrose (Sucr.), maltose (Malt.), and glucose (Glu.) B) Hydrolysis catalysed by α-amylase (0.0045 µL/mg). Carbohydrates are mainly degraded into olygomers of two glucose subunits or more. C) Hydrolysis catalysed by amyloglucosidase (0.00225 µL/mg). Carbohydrates are broken down into glucose units. D) Hydrolysis catalysed by α-amylase (0.0045 µL/mg) and amyloglucosidase (0.00225 µL/mg) combined. α-amylase initially hydrolyses carbohydrates into glucose dimers, which are then broken down into glucose monomers by amyloglucosidase. Microalgal lysate (20 mg of dry microalgae / mL) was heated at 90ºC for 5 min before addition of the enzymes. Reactions were carried out at 42.5ºC in 50 mM acetate, pH 4.6 buffer. Concentrations are shown as volume of added enzyme per mass of microalgal lysate. Time is shown in minutes, and ø corresponds to the microalgal extract before the addition of the enzyme.