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Development of a process for the production of L-amino-acids concentrates from microalgae by enzymatic hydrolysis

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Abstract

A process for the production of L-~~aminoacids~~amino-acids concentrates from microalgae biomass by enzymatic hydrolysis has been developed. The process includes pre-treatment for cell-disruption, enzymatic hydrolysis and final separation by centrifugation. Thermal and mechanical cell-disruption methods have been tested, selecting mechanical disruption using bead milling for 30 minutes ~~being selected~~. The enzymatic hydrolysis was done using the commercial enzymes Alcalase and Flavourzyme. Maximum ~~yield~~ hydrolysis was obtained for biomass concentrations under 270 g/l and previous additional treatment with Viscozyme, reaching a 42% hydrolysis. Repeated ~~hydrolysis~~reaction steps increased the hydrolysis ~~yield~~ from 42% (4 h) with a single step to 59% (8 h) after two successive ~~hydrolysis~~ steps. Further increase of the number of ~~hydrolysis~~ steps had a meagre impact on the global yield. The process widens the portfolio of products that can be obtained from microalgae biomass and is a new possibility to enhance the economic viability of microalgae-based biofuels production processes.

Keywords: microalgae biomass, free amino-acids concentrates, protein hydrolysis, enzymatic hydrolysis

1. Introduction

There is currently great interest on biofuel production from photosynthetic microorganisms, such as microalgae and cyanobacteria. It has been recurrently claimed that the production of microalgae can be economically and environmentally sustainable, and that hydrocarbon feedstock from algae biomass offers significant advantages over more conventional crops such as canola and soy bean (Chisti, 2007). For these reasons, technology is being developed and scaled-up to demonstrate the viability of the process. Technology must be scaled-up several orders of magnitude to make a significant contribution to the current biofuels ~~market,~~market; the technology now available must be scaled up by several orders of magnitude. For instance, to replace only a 5% of the US demand of fuel for transport it is necessary to produce more than 66000 kt/year of oil rich biomass (Chisti, 2007) while, ~~currently,~~ the microalgae biomass market produces only 5 kt/year (Pulz and Gross, 2004).

The oil content of microalgae biomass is commonly between 10 and 30%, but this upper limit is only obtained under stressing conditions, such as long storage time or intense deprivation of nutrients, not suitable for biomass production. Besides lipids, other major components in the microalgal biomass are carbohydrates (20-30%), ash (5-10%) and proteins (30-60%) (Rebolloso Fuentes et al., 2000, 2001). For high growth rate cultures, which are usually the most productive in term of biomass, proteins are the most abundant component in the biomass, representing more than 45% of the total dry weight, whereas lipids can be found as 20 to 30% of the biomass depending on which strain is used (Acién et al., 1998; Fernández et al., 2000). Therefore the production of large amounts of lipids from microalgae involves the production of a large deal of protein, which can in fact be expected to double the amount of lipids produced. So, it is clear that the protein production must be valorised to make the process economically positive.

Proteins from microalgae can be used for human and animal nutrition (Spolaore et al., 2006) but in both cases enzymatic hydrolysis is recommended to improve the digestibility of cell proteins as most microalgae are indigestible to monogastric animals and humans (Morris, 2008). In addition, hydrosylates can be useful for the production

of bacteria and yeast in the fermentation industry (Safari et al., 2009), as antioxidants (Sheih et al., 2009), or even used as energy source allowing the recovery of reduced nitrogen (Huo et al., 2011). Microalgae biomass has also been proposed as biofertilizer by its amino-acid profile and its content in other algae-derived natural substances (Ördög et al., 2004). Amino-acid -based fertilisation supplies plants with the necessary elements to develop their structures saving metabolic energy since the nitrogen does not have to be reduced as happens in the case of nitrate, which must be reduced to ammonium prior to its incorporation and conversion to α-ketoacids in order to synthesize ~~aminoacids~~amino-acids. It has been demonstrated that the application of ~~aminoacids~~amino-acids- in foliar (Jie et al., 2008) and soils (Mitchell et al., 1994) has a favourable effect on plants. By-products from meat and vegetal origin (pea meal, soya flour, sunflower) have been successfully tested to obtain those (Kubo et al., 1994; Ordóñez et al., 2001; Soetrisno and Holmes, 1992; Tejada and Gonzalez, 2003). Protein hydrosylates can be obtained by different methods such as acid hydrolysis, with exogenous proteases, or enzymatic autolysis. Acid hydrolysis gives a higher yield, but the obligatory neutralization step results in a high ash content, which limits the applicability of the product (Ovissipour et al., 2009).

This paper focus in the development of a process for the production of free-~~aminoacids~~amino-acids concentrates from ~~microalgal~~microalgae biomass. The method is based on the enzymatic hydrolysis of proteins contained in the biomass. The enzymatic reaction step has been optimized in addition to the pre-treatment step necessary to improve the yield of the process. Enzymatic hydrolysis is selected instead chemical hydrolysis to produce a high quality product, rich in essential ~~aminoacids~~amino-acids, free of toxic degradation-products because the process is carried out under mild conditions.

2. Materials and methods

2.1. Microalgae biomass, enzymes and chemicals

The raw material used for the experiments was dry-~~freezed~~freeze biomass of the freshwater strain *Scenedesmus almeriensis*. This strain was isolated from a fresh water pool in a greenhouse located in Almería (2º43'W, 36º48'N), SPAIN. Cell size is 3 µm

diameter by 6 µm large, oval morphotype, and its growth and composition characteristics has been previously studied. This strain has been deposited in the Culture Collection of Algae and Protozoa as Scenedesmus almeriensis CCAP276/24. The biomass was produced in continuous mode at 0.3 1/day in an industrial size tubular photobioreactors (3 m^3) at Estación Experimental Fundación Cajamar located in Almería, Spain. The biomass was produced in summer time, and because the photobioreactors used allows controlling the culture parameters, it was produced at 20-28°C and pH ranging from 7.8-8.2, using Mann and Myers medium prepared using commercial fertilizers~~-~~. The biomass was harvested and centrifuged daily, and then it was freeze-dried to preserve it. To determine the proteins content of the biomass the Lowry method was used. Lipids were determined as the extract obtained with chloroform:methanol (2:1) (v/v) (Kochert, 1978). Fatty acids were determined by gas chromatography, as it describes Rodriguez Ruíz et al., (1998). Carbohydrates were estimated by the anthrone spectrophotometric method (Osborne, 1986). Total ash was determined by incineration of a representative 0.5 g sample in an oven at 450ºC for 48 h. The biochemical composition of the biomass was: 41.8% proteins, 11.2% lipids (8.5% fatty acids), 38.7% carbohydrates, and 8.3% ash.

The enzymes used were supplied by Novozyme A/S (Bagsvaerd, Denmark). These enzymes are commercial enzymes used for the production of amino-acids hydrolysates, from yeast and other substrates, which are finally commercialized as fertilizers. The hydrolysis a~~e~~ssays were done with Alcalase 2.5L (2.5 AU-A/g) and Flavourzyme 1000L (1000 AU-A/g). The enzyme Viscozyme L (50000 U/g) was used to decrease the viscosity of the reacting mixture in order to enhance the yield of the reaction. This enzyme is a betaglucanase-cellulase-xylanase commercialised for the reduction of viscosity in fermentation media being widely used at industrial scale. The chemicals used for analytical methods were sodium tetraborate decahydrate, DL-Dithiothreitol, Phthaldialdehyde, Dodecyl sulphate and DL-Serine ~~were~~ obtained from SIGMA-ALDRICH. Also ethanol, Albumin Bovine (BSA), Copper(II)sulfate-pentahydrate, Dodecylhydrogensulphate Sodium Salt (SDS), Folin-Ciocalteau´s phenol reagent, Potassium Sodium Tartrate tetra hydrate, Sodium carbonate, Sodium hydroxide and Sulfuric acid from Panreac ~~were~~ was used.

2.2. Pre-treatment of the biomass

Pre-treatment of the biomass by thermal and mechanical cell-disruption methods was studied. Thermal pre-treatment was performed in microalgae pastes of 200 g/l concentration. Samples of 200 ml were heated at 80ºC, under mild agitation using magnetic stirrers, for process times ranging from 0 to 20 min. The solution was cooled in a water bath and then the enzymatic hydrolysis was immediately performed at normalized conditions (4%v/v Alcalase 2.5L, two hours ~~time~~, pH =8.0, 50º°C). To carry out the mechanical pre-treatment, dry-freeze biomass was mixed with different proportions of alumina to make up 200 g samples of mixtures biomass/alumina which were each treated in a horizontal-bed ball-mill (20 cm radius at 40 rpm, 2 cm balls). Different milling times were tested for each biomass-to-alumina ratio. After the milling, the sample was mixed with water to form ~~a 200 g/l paste, as described before, which were~~a 200 g/l paste, as described before, which was the substrate of the enzymatic hydrolysis under normalized conditions.

2.3. Enzymatic hydrolysis method

Enzymatic hydrolysis experiments were performed in a batch reactor of 9 cm diameter and 2:1 height to diameter ratio, equipped with temperature and pH control. Agitation was supplied by a 3 cm diameter Rushton turbine. The experiments were performed on the above mentioned microalgae biomass suspensions in two stages, each at the optimal conditions recommended by the enzyme suppliers. In the first stage the biomass is transferred into the hydrolysis reactor and heated to 50ºC. The pH is adjusted to 8.0 by adding NaOH (1M), then Alcalase 2.5L is added first and the reaction begin. Free ~~aminoacids~~amino-acids are released as the reaction proceeds, which acidifies the medium tending to decrease the pH. NaOH 1M is added on demand by using a low volume diaphragm pump to maintain the pH at 8.0. In the second stage the pH is lowered to 7.0 by adding H_2SO_4 1 M and then the second enzyme Flavourzyme 1000L is added. The pH is maintained at 7.0 during this period by adding H_2SO_4 (1M) also using a low volume diaphragm pump. The volume of NaOH-H_2SO_4 added allows measuring the progress of the reaction because it modifies the pH, although it is not a precise measurement of conversion taking ~~.~~place. After the

reaction is finished (the degree of hydrolysis do not improves), the solution is heated to 75ºC for 15 min to deactivate the enzymes. The final concentrate rich in free ~~aminoacids~~amino-acids is separated from the waste biomass by centrifugation at 8000 rpm in a discontinuous centrifuge (Meditronic. J.P.Selecta, Spain).

2.4. Protein content and hydrolysis degree

The protein content of samples was determined using a modification of the Lowry method (López et al., 2010), with BSA as standard. The hydrolysis degree, defined as the ratio between the number of free amino-acids and the total number of amino-acids-protein available for hydrolysis. The hydrolysis degree was measured using the OPA method with serine as standard (Nielsen et al., 2001). This method is based on the reaction of ortofenilaldehide (o-phthaldialdehyde) (OPA) with free α-amine groups giving a derivative that can then be measured spectrophotometrically at 340 nm to quantify the amount of free α-amine groups in the sample. According to this method the following values of the parameters of the model have been selected α=1, β=0,4 y h_{tot}=8 (Nielsen et al., 2001).

2.5. Statistical analysis

Experiments were performed at least by triplicate, mean values and standard deviation being showed. Statistical analysis of data was carried out with the software Statgraphics version 5.0.

3. Results and discussion

Protein hydrosylates can be produced ~~both chemically or~~both chemically and enzymatically, but enzymatic hydrolysis gives higher quality products (Ovissipour et al., 2009). Concerning enzymatic hydrolysis, the correct choice of enzymes and the optimization of the operating conditions is crucial for the success of the process. Although there is a wide portfolio of enzymes available commercially for a variety of transformations and with suggested operating conditions, the application of those enzymes to any new raw material or substrate requests a reconsideration of the process variables due to the specificity of enzymes to both substrate and

environmental conditions. In this sense the production of free-~~aminoacids~~amino-acids concentrates from microalgae biomass requires three steps: the solubilisation of the proteins, the hydroxylation it and the separation of the product from the waste biomass. To solubilise the proteins contained into the microalgae biomass it is necessary to break the cells walls by any of the different possible treatments. Here, the chemical treatments such as alkaline or acid attacks have been avoided in order to preserve the quality of the product as much as possible. Therefore the thermal and mechanical disruption methods have been chosen. The experiments were performed applying different thermal and mechanical treatment intensities to the raw material. The resulting biomass was subjected to enzymatic hydrolysis with Alcalase 2.5L in order to evaluate possible adverse effects of the enzymatic reaction.

The results show that both disintegration methods, heating and milling, improve the degree of hydrolysis obtained after the enzymatic treatment (Figure 1). The influence of milling was more noticeable, attaining an increase of the hydrolysis degree from 13% to 50% after 35 minutes milling. On the other hand, the thermal treatment only increased the degree of hydrolysis to 30% after 12 min of treatment, decreasing for longer heating times. Regarding the milling operation, which was performed with alumina added to the biomass in a 50% d.wt. proportion, one concern is if it is possible to reduce the amount of alumina without decreasing the efficiency of the operation. The experiments done to check this show that high proportions of microalgal biomass to alumina in the sample (lower alumina content) resulted in an improved yield of the enzymatic reaction, reaching a maximum value of 50% with no alumina added (Figure 2). Since alumina help to break cells it cannot physically hamper the hydrolysis process, therefore the reduction in the hydrolysis degree observed when the alumina percentage increases must be due to a chemical interaction with either the enzyme or the reacting proteins. The optimization of the pre-treatment step was completed with an experiment that combined the thermal and mechanical treatments under the optimal conditions found separately. The data obtained show that hydrolysis degree of thermal treatment alone (80ºC for 12 min) was 31% while milling alone (30 min without alumina) resulted in a maximum of 50% hydrolysis. The combination of both

methods gave the same result as milling alone, 50% hydrolysis, thus the thermal treatment can be discarded.

Once the pre-treatment is optimized, the enzymatic hydrolysis step comes next. The optimal timing for the addition of the enzymes was obtained first by monitoring the evolution of free ~~aminoacids~~amino-acids in the reaction mixture. Alcalase 2.5L, an endoprotease, was used first under the recommended operating conditions a temperatures of 50ºC and pH=8. As the enzyme breaks down the proteins free-~~aminoacids~~amino-acids are released, increasing in concentration for 120 min. After this time the degree of hydrolysis remained constant. At this point Flavourzyme 1000L, an exoprotease, was used in an attempt to continue the hydrolysis. This enzyme shows optimal activity at 50ºC and pH=7. The reaction mixture conditions were adjusted to meet the new recommended optimum conditions. After using Alcalase 2.5L, the conditions of the reacting mixture were modified and Flavourzyme 1000 L added. This increased the degree of hydrolysis from 48 to 59% after 180 to 230 min of continued experiment. Thus, the optimal time recorded for each enzyme was 120 min for Alcalase 2.5L alone, plus 60 min for Flavourzyme 1000L, for maximum hydrolysis degree, totalling a enzyme hydrolysis step 3 h long.

Biomass and enzyme concentration during the enzymatic hydrolysis are other important operating parameters and thus have to be optimized. Enzymes are costly and are used in concentrations as low as possible, which are normally between 1 and 5% in this type of application. Since enzymes are catalysts, under normal conditions (no inhibition or other adverse phenomena) the rate of the catalysed reaction is proportional to the enzyme concentration. This was verified experimentally (data not shown) and an enzyme concentration of 4% was selected as a compromise between operation time and reaction rate for the rest of the experiments. With regard to the biomass concentration, it is obvious that the higher the concentration used the more amount processed per batch but it is limited by factors such as (i) the rheological properties of the raw material, (ii) the tolerance of the process to high biomass concentrations, and (iii) the final product to be obtained. To obtain a product with a suitable concentration of free-~~aminoacids~~amino-acids it is desirable to use as high biomass concentration as possible while achieving high hydrolysis degrees. Since this

work aims at an industrial application, the minimum biomass concentration used in this research was 200 g/L, which corresponds to the lower range of biomass concentration of the microalgae biomass sludge obtained by continuous centrifuges. More intense centrifugation or the use or other harvesting techniques such as filtration can result in a more concentrated microalgae biomass paste, with as much as 350 g/l of dry matter. To determine the influence of dry matter content of the raw material in the enzymatic hydrolysis step experiments modifying this parameter in the referred range were carried out.

The experiments show that when the biomass concentration increases over 200 g/l the yield of enzymatic reaction is decreased and the degree of hydrolysis measured is lower (Figure 3). The hydrolysis degree drops from 55% at 200 g/l to 20% at 350 g/l. Consequently the free-~~aminoacids~~amino-acids concentration obtained is lower when the biomass concentration is increased. The free-~~aminoacids~~amino-acids concentration decreases from 47 g/l to 29 g/l in the described experiments. These data indicates that in spite of the highest protein concentration in the culture when the biomass concentration is higher, the reduction in the yield of the enzymatic hydrolysis predominates, resulting in a lower final concentration of free-~~aminoacids~~amino-acids when biomass concentration increases. To improve the free-~~aminoacids~~amino-acids concentration in the final product it is necessary to improve the yield of the process at the highest biomass concentration as much as possible. For this, additional experiments were performed adding the enzyme Viscozyme in order to reduce the viscosity of the solution as a means of enhancing mass transfer to help to increase the yield of the enzymatic hydrolysis. Viscozyme is an enzyme with activity betaglucanase-cellulase-xylanase thus breaking carbohydrates and reducing their influence in the enhancement of the viscosity of medium. The treatment with Viscozyme was done at 50ºC for 30 min. The results show that the optimal performance was obtained using Viscozyme at pH between 6 and 7, displaying a decrease in the yield of hydrolysis for more acidic conditions (Figure 4). The use of Viscozyme increased a 30% the yield of the process and so this step was included as a part of the final process.

The free-~~aminoacids~~amino-acids concentrates obtained in the enzymatic hydrolysis process proposed in this work are of high interest as fermentation medium

(Safari et al., 2009), biofertilizers (Ördög et al., 2004), antioxidant (Sheih et al., 2009) and even as energy source (Huo et al., 2011). Other relevant market for this product can be as fertilizer for foliar (Jie et al., 2008) and soil (Mitchell et al., 1994) applications in plants. After the withdrawal of most of the proteins by the proposed process, the lipid and carbohydrate content of the remaining biomass increases, which facilitates the next extraction steps by eliminating potential interference, enhancing its yield. Moreover, the cell disruption following protease treatment increased lipid extractability (Kechaou et al., 2009). Thus, cell-wall disruption of *Chlorella* sp. using proteases increased the yield of lipid extraction from 32 to 56% (Fu et al., 2010).

The possibility of improving the utilization yield of the protein fraction in the microalgal biomass by performing a repeated enzymatic hydrolysis step on a sample was also checked. For this, Alcalase 2.5L and Flavourzyme 1000 L were added consecutively as described in the standard procedure. The temperature was set at 50ºC and the pH was adjusted to 8.0 during the action period of Alcalase 2.5L, and then to 7.0 for the addition of Flavourzyme. Data show that carrying out a second hydrolysis step increases the hydrolysis degree 8%, from 51% to 59% (Figure 5). Further repetitions were analogously carried out but the results obtained were poor. The global degree of hydrolysis increased to 64% in the third hydrolysis and to 66% in fourth repetition, respectively. According to these data we decides to adopt a two-step hydrolysis as standard procedure although optimal number of extraction step in a given purification process should be assessed by an economical analysis.

The selected enzymes Alcalase and Flavourzyme have been widely used to hydrolyse proteins from vegetal origin (Tang et al., 2009), animal sources (Camacho et al., 1998) and from fish by-products (Kechaou et al., 2009; Ovissipour et al., 2010). In those applications the degree of hydrolysis obtained was very low in cases. For instance, when used on fish viscera the hydrolysation yield was only 7% (Kechaou et al., 2009). In the hydrolysis of protein from hemp seed (*Cannabis sativa*) the highest hydrolysis yield obtained using Alcalase was 40%, whereas the use of Flavourzyme only yielded a 10% (Tang et al., 2009). In both cases the reaction is nearly finished at 120 min (Tang et al., 2009). With regard to the length of the chains of the peptides obtained from the hydrolysis process, it has been reported that the product is mostly

free ~~aminoacids~~amino-acids at degrees of hydrolysis higher than 20%, instead of the various length polypeptide chains found at lower hydrolysis grades (Gonzalez-Tello et al., 1994). The data here reported showed hydrolysis degrees up to 59% when both enzymes were combined as described and for two repeated steps. Other authors report substantially lower yields when working with microalgae. Morris el al. (Morris et al., 2008) obtained a maximum hydrolysis degree of 20% working with *Chlorella* sp even with a previous extraction of the protein fraction using ethanol, which dropped to a 10% yield if the hydrolysis was done without previous extraction. This ~~low~~ yield of enzymatic hydrolysis working with *Chlorella* sp. could be due to their thick cell wall or to a poor choice of adequate enzymes.

To achieve a high degree of hydrolysis it has been necessary to develop and optimize a complete process for the enzymatic hydrolysis of proteins contained into the biomass. The optimization of the cell-disruption step suggested that milling for 30 min was an optimal pre-treatment of *S. almeriensis* freeze-dried biomass. This process time could differ if other strain is used, according to cell-robustness. The utilization of fresh biomass instead of freeze-dried biomass might also need a modification of the cell-disruption step. Experiments performed using fresh biomass with the same conditions here identified as optimum resulted in higher hydrolysis degrees than those found for freeze-dried biomass. This shows a clear advantage of using fresh biomass, which adds to the benefit of avoiding the drying step, thus reducing the global processing cost of the biomass, whereas pre-treatment of cell-disruption can be done using homogenizers. Concerning the enzymatic reaction step, at this point it has been optimized with the objective of maximizing the hydrolysis degree, disregarding the economical aspects. However, to obtain the economical optimum, the process must be optimized as a whole, combining the enzymatic hydrolysis with the production and harvesting of the biomass. In the diverse microalgae culture systems currently in use, the biomass concentration usually varies between 0.2 to 2.0 g/l, thus to obtain microalgae biomass concentrates suitable for the process here described, a considerable energy expenditure is required, increasing proportionally to the final concentration required. To obtain ~~aminoacids~~amino-acids concentrates of concentration high enough as to be considered of commercial interest it is necessary

to process highly concentrated biomass. The highest free-~~aminoacids~~amino-acids concentration achieved in a single enzymatic hydrolysis step was 47 g/l, increasing to 55 g/l for two steps (at the cost of doubling the consumption of enzymes and time). Using *Chlorella* sp, a maximum free ~~-aminoacids~~amino-acids concentrations of 39 g/l were obtained, decreasing substantially when processing biomass concentrations over 200 g/l (Morris et al., 2008). Only an economical analysis of the overall production process of free-~~aminoacids~~amino-acids concentrates, including biomass production step and downstream operations to produce other co-products (bioethanol, biodiesel, etc.), will allow optimizing the entire process.

The process developed in this work is highly interesting to clarify the biorefinery scheme of the production of biofuels from microalgae. Oil prices and global warming issues derived from the use of fossil fuels are strong incentives driving the search to find alternative sources of energy. The photosynthetic production of biofuels represents one of the most attractive because those processes rely on completely renewable resources: sunlight as energy source and CO_2 as carbon source. The biomass produced can be transformed into biofuels via fermentation of the starchy/sugary fractions to produce bioethanol, or via lipid processing (eg. transesterification) to produce biodiesel. Alternatively the biomass can be digested to produce biogas. Being a commodity needed in such large quantities, suitable conversion processes for the conversion of biomass into biofuels must have a very high yield turning sugar/starch and lipids into bioethanol and biodiesel (Anemaet et al., 2010), or the wastes from those processes must be suitable to be further processed by, for instance, a digestion step to produce more energy (Heaven et al., 2011). Otherwise the wastes would unmanageable. However, all these schemes disregard the major fraction of the biomass, proteins.

Proper use of the protein fraction is a must for a feasible biofuels production process. Moreover, proteins can interfere in the downstream sequence of operations necessary to produce bioethanol or biodiesel by adding bulk to the process stream or facilitating parallel, unwanted chemical reactions with other components. Therefore the inclusion of a step that allows removing the proteins from the microalgae biomass and turning them into valuable products is very desirable. According to data and

operations here reported, a block diagram summarising the process has been proposed (Figure 6). According to the proposed process the cells must be disrupted mechanically previously to be processed. Next, the biomass, at maximum biomass concentrations of 270 g/l, is treated with Viscozyme to reduce its viscosity. Then it can be subjected to enzymatic hydrolysis using Alcalase 2.5 L and Flavourzyme 1000L, twice for 3 hours period. Once the reaction has been performed the enzymes can be deactivated by heating at 75ºC for 15 minutes. The supernatant containing the free-~~aminoacids~~amino-acids concentrate can be finally separated from the waste biomass, with low nitrogen content, by centrifugation. The quality of the product obtained with the process described here has been ~~evaluated~~ determined by HPLC in an external reference laboratory. Its composition in free-~~aminoacids~~amino-acids is shown in Table 1. Although the t~~T~~he major ~~aminoacids~~amino-acids were nonessential amino-acids as glutamine, serine and glycine, with contents higher than 10% of total amino-acids, the product contain essential amino-acids at percentages higher than 5% as histidine and lysine, and minor quantities of others. ~~serine and glutamine, following glicine and glutamic acid.~~ Only aspargine, tr~~y~~yptophan and h~~y~~droxy~~i~~poline were below 0.16%. By comparing the amino-acids profile of obtained product with obtained from other sources as fish viscera it is observed that serine, glutamine and histidine content is higher in hydrolysate from *Scenedesmus* than from fish viscera, whereas alanine and aspartic acid content are lower (Kechaou et al., 2009). It is especially noticeable that the hydrolysate from *Scenedesmus* contain more than 7% of histidine, this amino-acid being not present in the hydrolysates from fish viscera. The product is transparent and free of particles, and it is been now studied in different application test. The development of this process widens the portfolio of co-products that can be obtained during the production of biofuels from microalgae.

4. Conclusions

The process presented here constitutes a realistic possibility to turn the protein fraction of microalgal biomass into a profitable product. Proteins, always a major fraction of microalgae biomass, have been disregarded for the most part in the numerous attempts at designing biofuels processes from microalgae. An alternative such as the one presented here, capable of processing input streams over 250 g/l

biomass concentration, with a yield close to 60% to produce ~~aminoacids~~amino-acids concentrates over 50 g/l, is probably an interesting addition to the portfolio of processing alternatives in the biofuels from microalgal biomass industry.

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Figure captions

Figure 1.- Influence of (A) time of heating (80ºC) and (B) ~~molturation~~ milling by ball-mills in the ~~yield~~ degree of ~~of enzymatic~~ hydrolysis of *S. almeriensis* biomass under standard conditions.

Figure 2.- Influence of biomass percentage in the sample (biomass-alumina) to be milled in the ~~yield of enzymatic~~degree of hydrolysis of *S. almeriensis* biomass under standard conditions.

Figure 3.- Influence of biomass concentration in the hydrolysis degree and free-~~aminoacids~~amino-acids concentration after enzymatic hydrolysis of *S. almeriensis* biomass under standard conditions.

Figure 4.- Influence of pH treatment with Viscozyme in the ~~yield~~ degree of ~~the enzymatic~~ hydrolysis of *S. almeriensis* biomass under standard conditions.

Figure 5.- Variation of ~~hydrolysis~~ degree of hydrolysis with the number of enzymatic hydrolysis repetitions performed over the same sample, previous separation of free-~~aminoacids~~amino-acids produced.

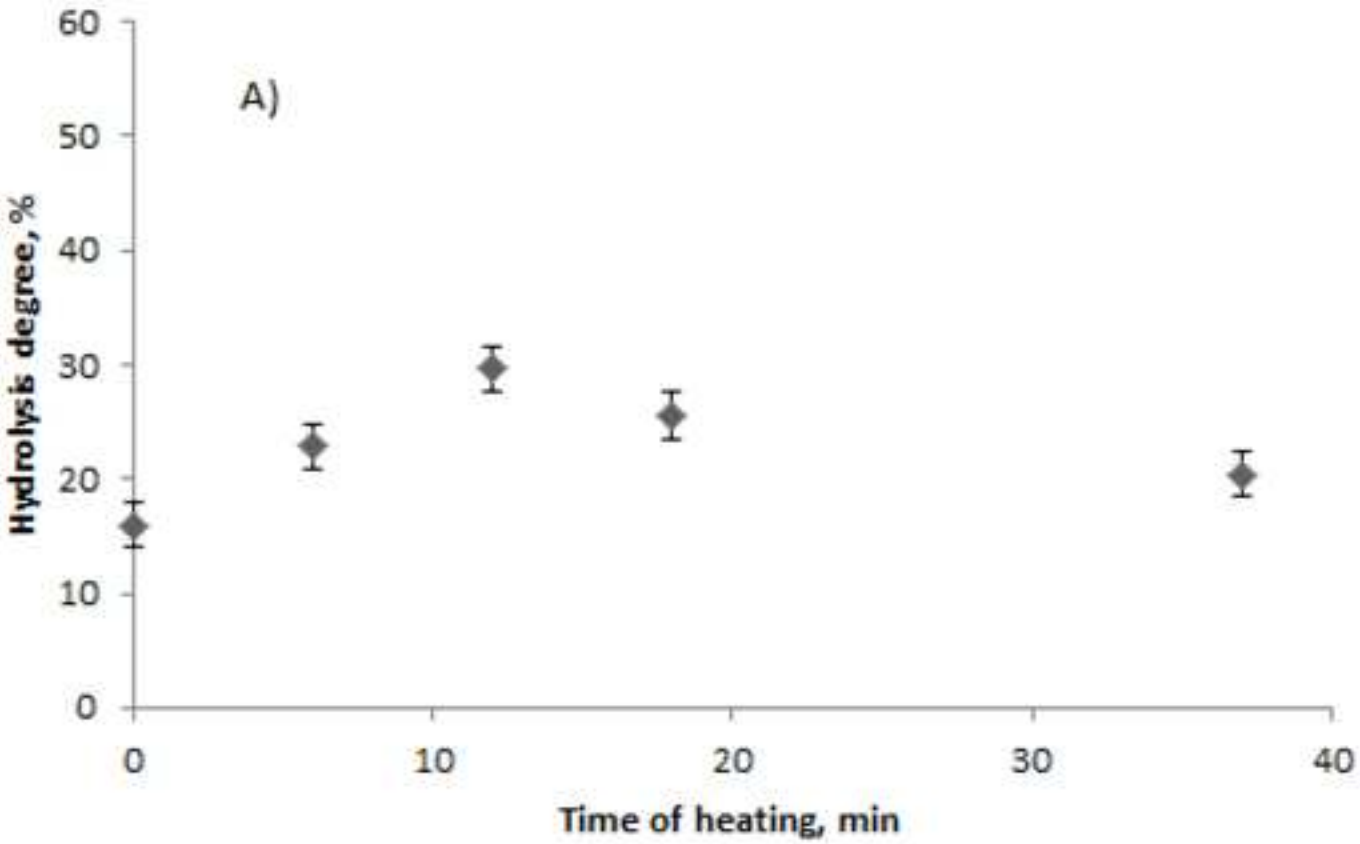
Figure 6.- Block-diagram of the proposed process for the production of free-~~aminoacids~~amino-acids concentrates from *S. almeriensis* microalgae biomass.

Table

Table 1.- Free-aminoacids composition of final product obtained.

Essential	Percentage, %	Nonessential	Percentage, %
Histidine	7.03	Glutamine	16.88
Lysine	5.47	Serine	16.25
Valine	4.69	Glycine	10.94
Isoleucine	4.53	Glutamic acid	7.19
Threonine	3.13	Proline	5.78
Phenylalanine	2.5	Arginine	5.63
Leucine	2.19	Tyrosine	3.44
Methionine	1.09	Alanine	1.88
Tryptophan	<0.16	Aspartic acid	1.41
		Asparagine	<0.16
		Hydroxyproline	<0.16

Figure 1


A)
Hydrolysis degree, %
60
50
40
30
20
10
0
0
10
20
30
40
Time of heating, min

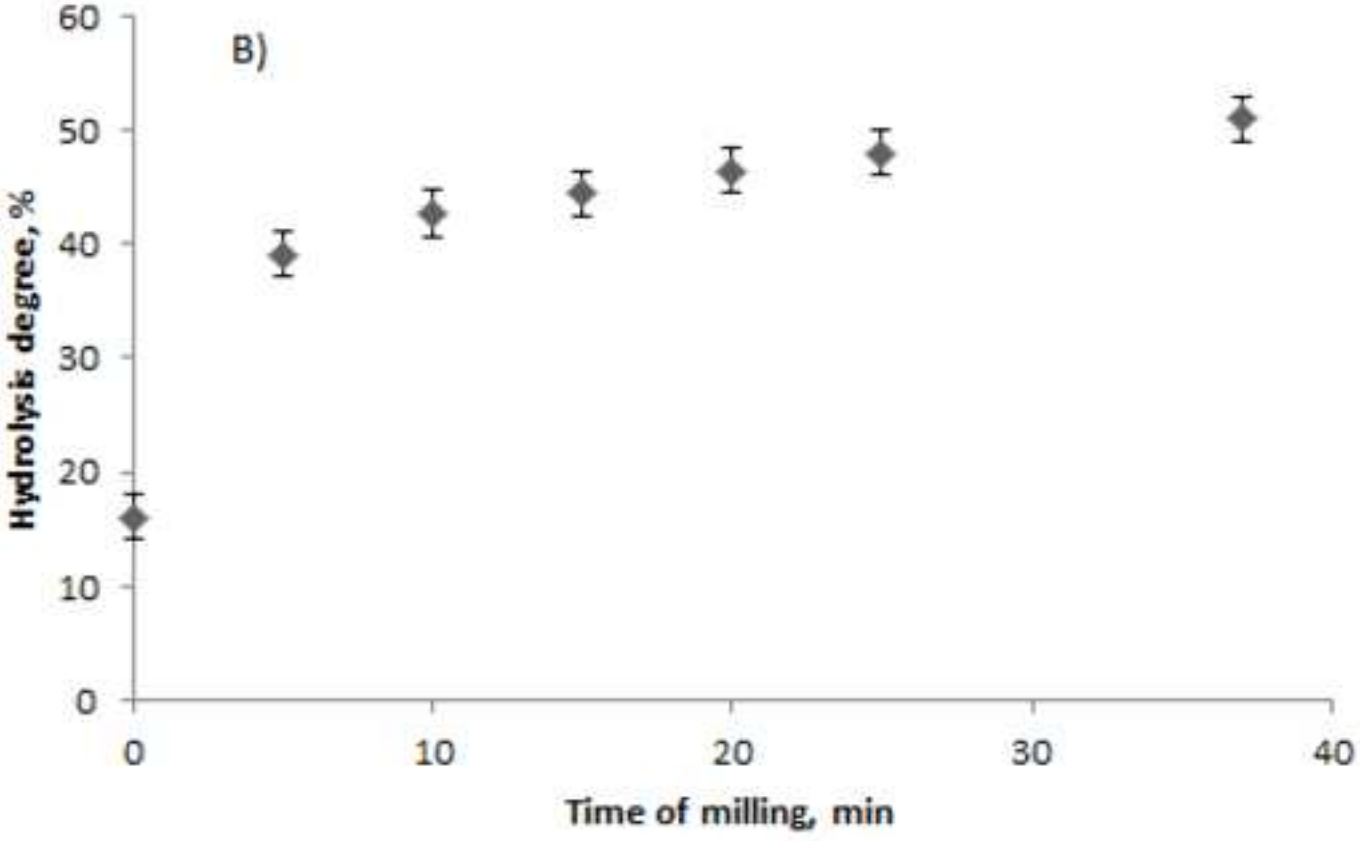

B)
Hydrolysis degree, %
60
50
40
30
20
10
0
0
10
20
30
40
Time of milling, min

Figure 2

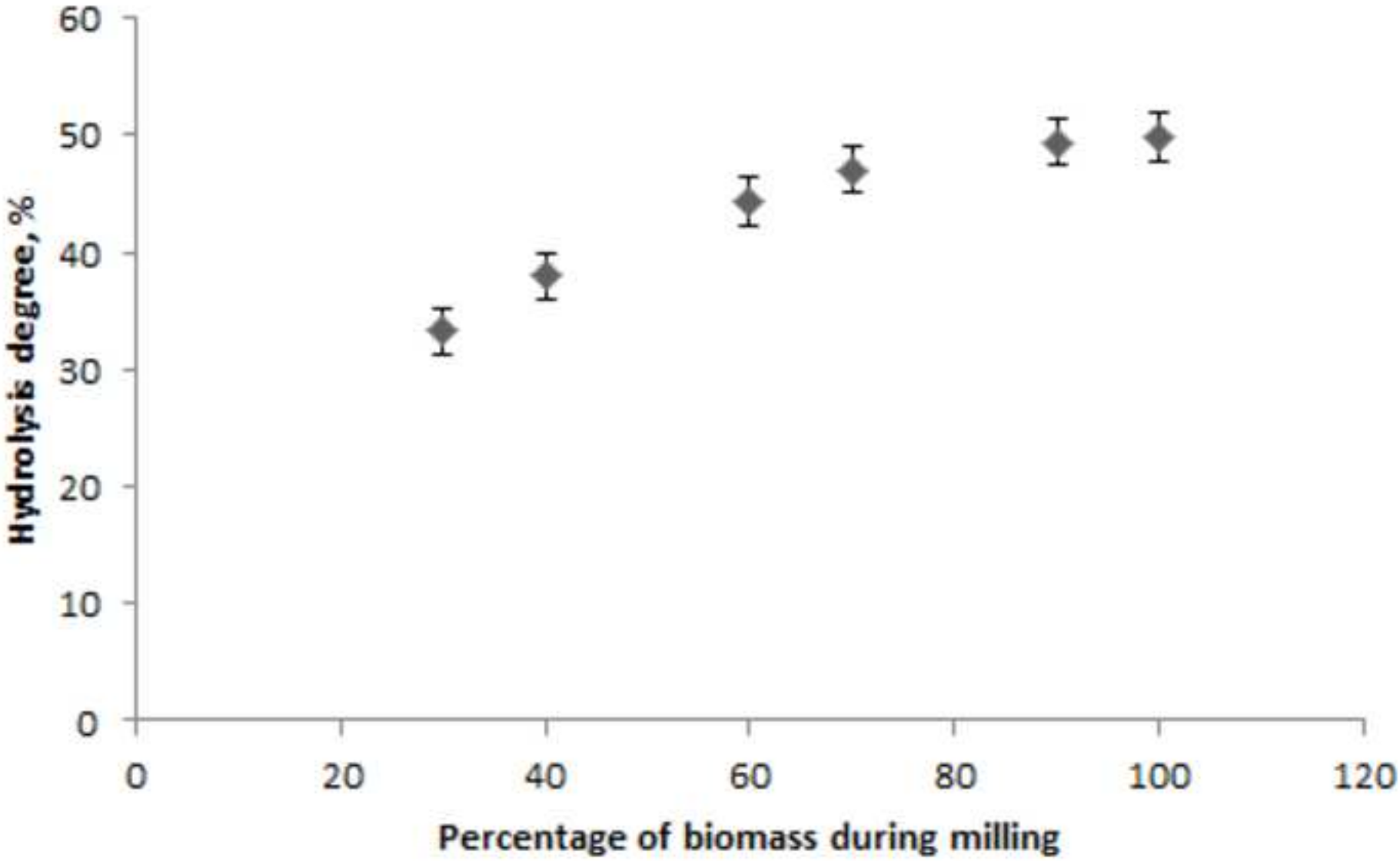

Hydrolysis degree, %
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Percentage of biomass during milling

Figure 3

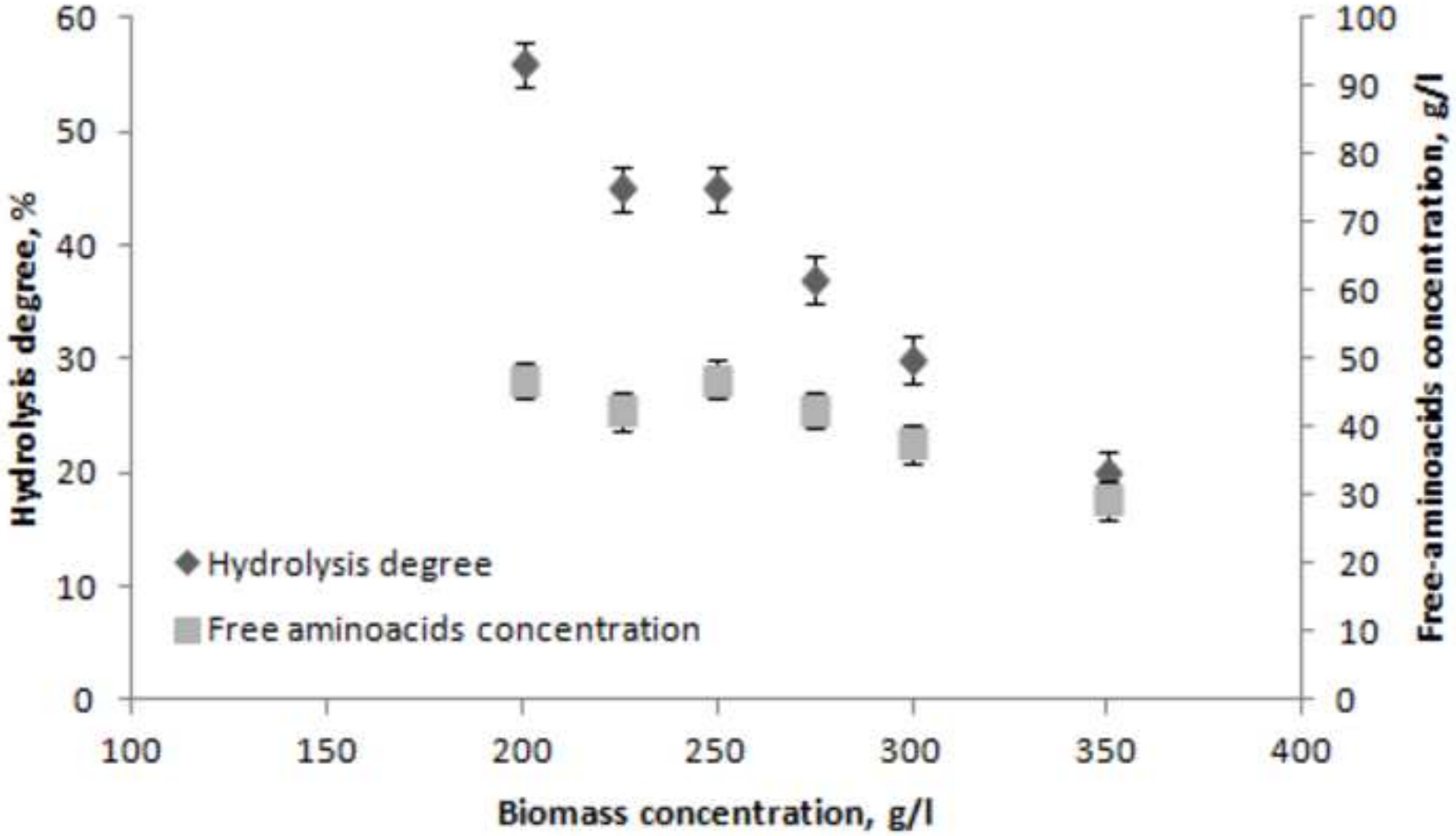

Hydrolysis degree, %
Free-aminoacids concentration, g/l
Biomass concentration, g/l
Hydrolysis degree
Free aminoacids concentration
0
10
20
30
40
50
60
70
80
90
100
100
150
200
250
300
350
400

Figure 4

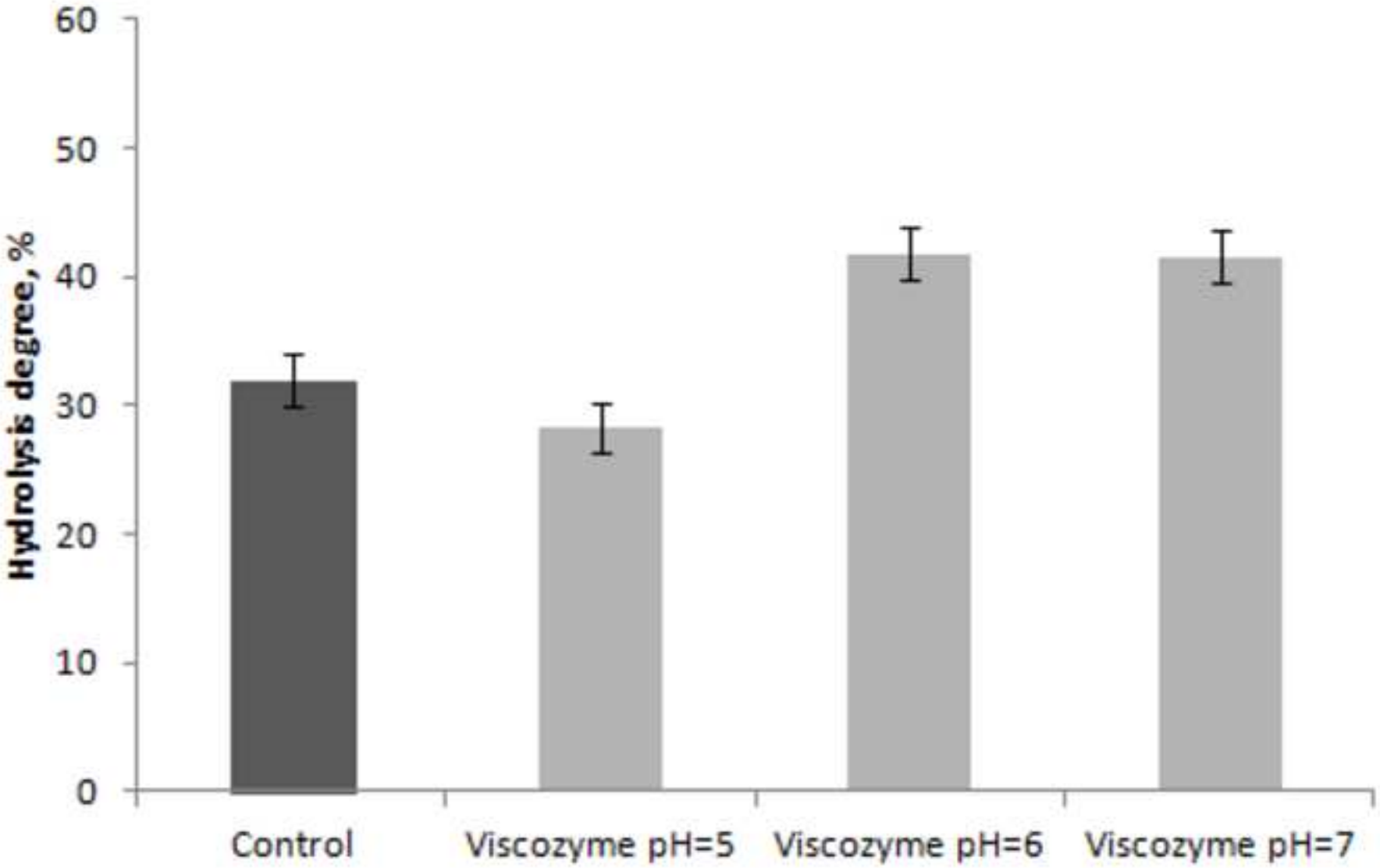

Hydrolysis degree, %
60
50
40
30
20
10
0
Control
Viscozyme pH=5
Viscozyme pH=6
Viscozyme pH=7

Figure 5

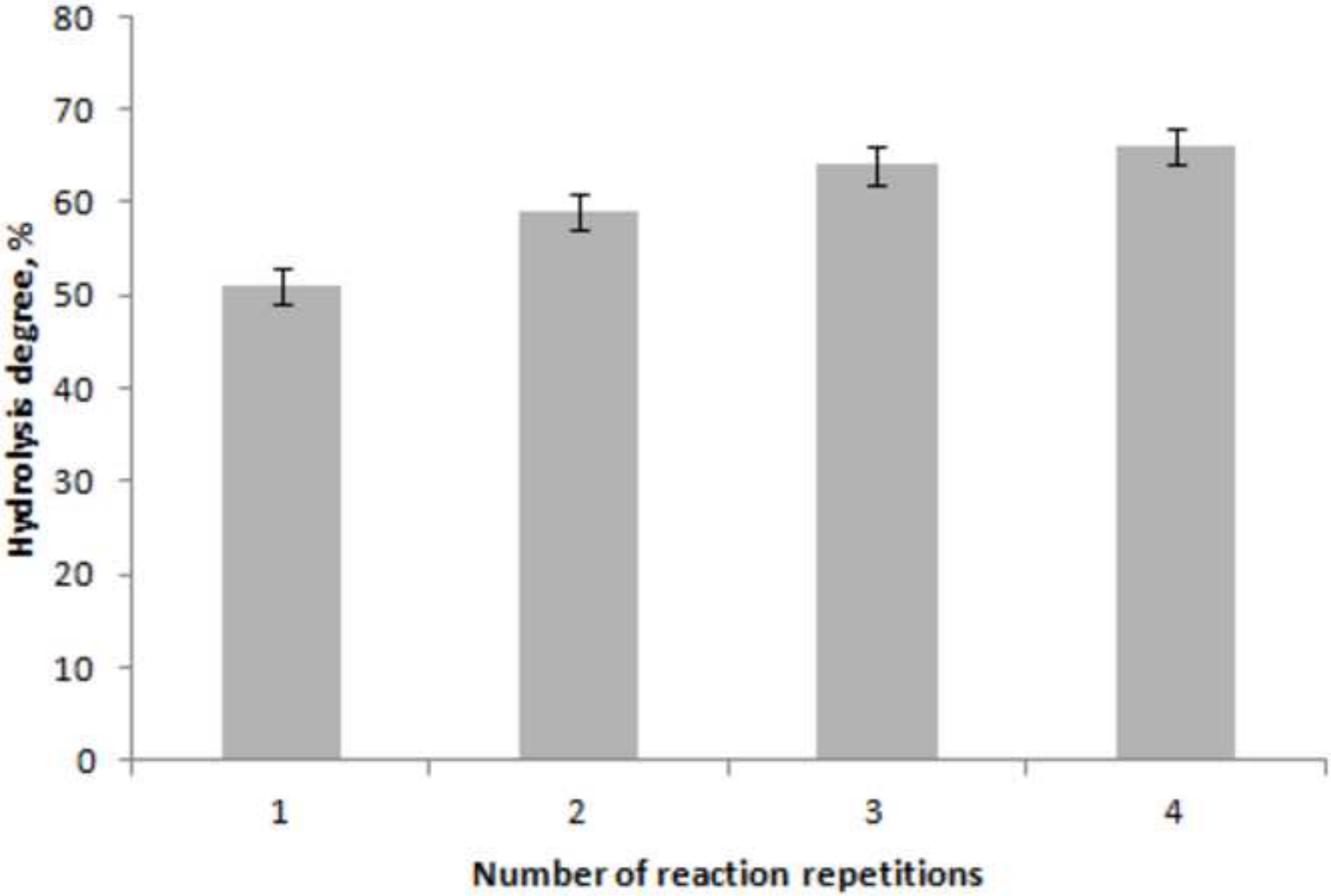
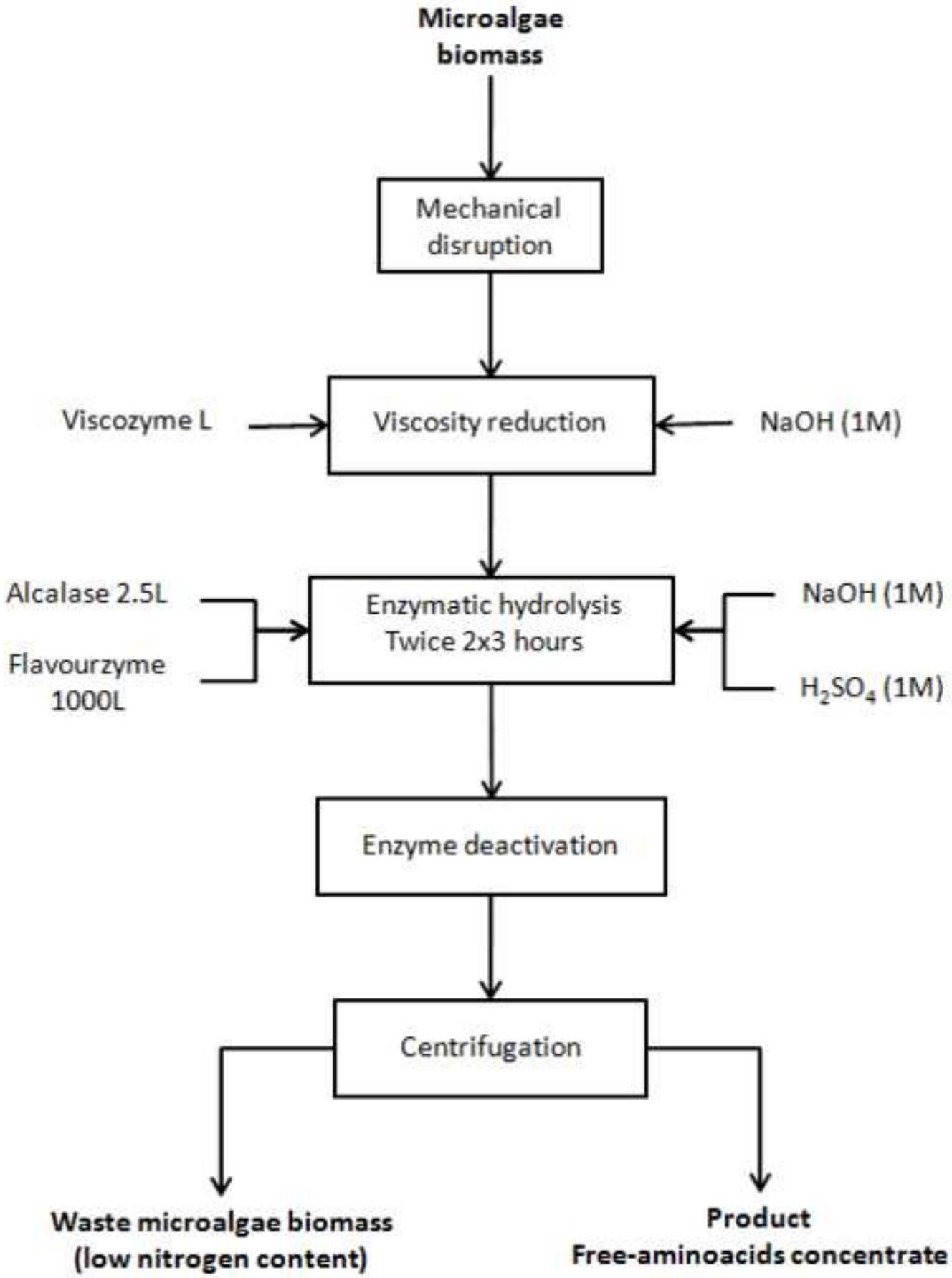

80
70
60
50
40
30
20
10
0
Hydrolysis degree, %
1
2
3
4
Number of reaction repetitions

Figure 6


Microalgae biomass
Mechanical disruption
Viscozyme L
Viscosity reduction
NaOH (1M)
Alcalase 2.5L
Flavourzyme 1000L
Enzymatic hydrolysis Twice 2x3 hours
NaOH (1M)
H_2SO_4 (1M)
Enzyme deactivation
Centrifugation
Waste microalgae biomass (low nitrogen content)
Product Free-aminoacids concentrate