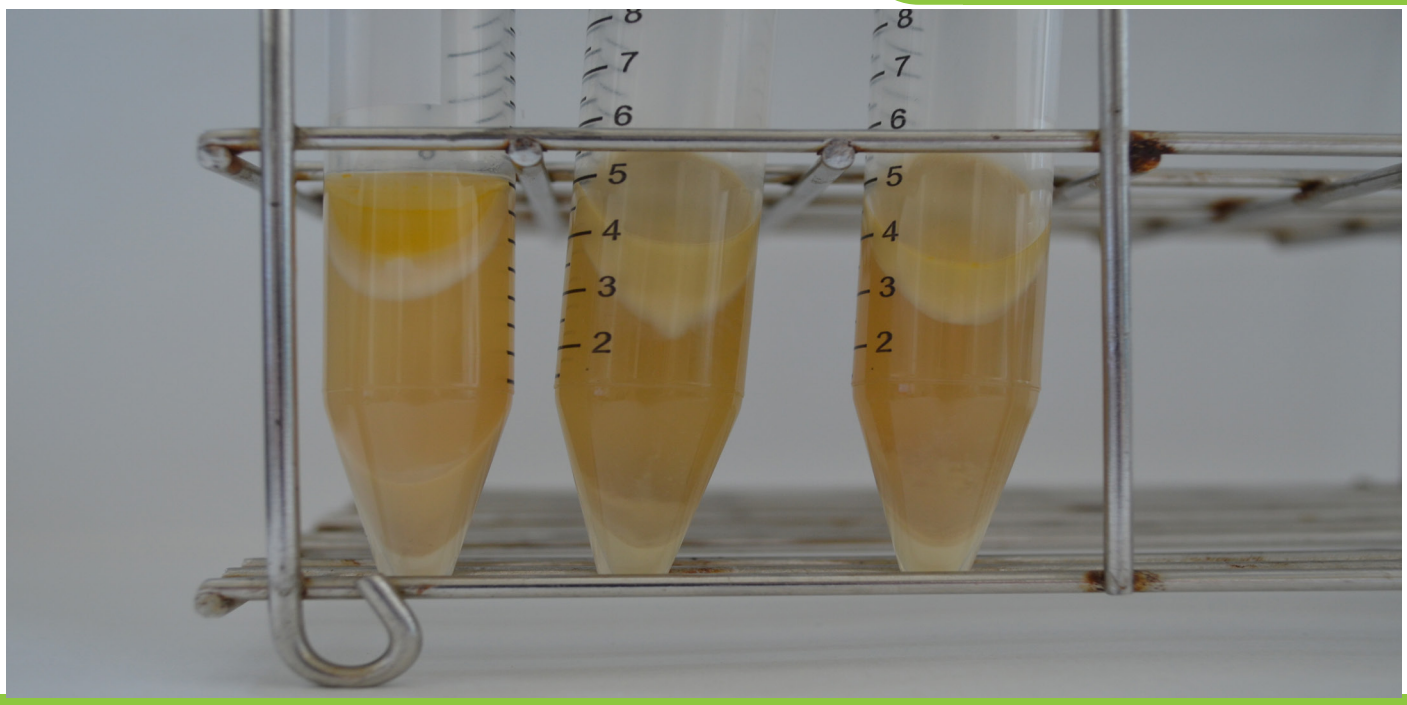
Interreg
North-West Europe
EUROPEAN UNION

ALG-AD

European Regional Development Fund

THEMATIC PRIORITY

RESOURCE AND MATERIAL EFFICIENCY



TECHNICAL REPORT: OPTIMISATION OF ENZYMATIC HYDROLYSIS

ENZYMATIC HYDROLYSIS FOR EXTRACTION OF OILS RICH IN N-3 PUFAS AND BIOACTIVE PEPTIDES FROM A BIOMASS OF HETEROTROPHIC PROTISTS (THRAUSTOCHYTRIDS).

EDITION : NOVEMBER 2020

Corresponding Authors

Mariana Ventura (Lead author, mariana.ventura@univ-brest.fr;

Philippe Soudant (soudant@univ-brest.fr);

Fabienne Guérard (fabienne.guerard@univ-brest.fr);

Acknowledgements

The authors would like to thank Louise Hall for proofreading and formatting this document; Antoine Bidault and Fabienne Le Grand for lipid analysis; Sylvain Petek for the help in SEC-HPLC analysis ; Cloé Oroy and Estelle Le Bihan for their advice in enzymatic hydrolysis

Please cite this document as follow:

Ventura, M., Soudant, P, Guérard, F. 2020. Optimization of an enzymatic hydrolysis process for the extraction of oils rich in n-3 PUFAs and bioactive peptides from a biomass of heterotrophic protists (Thraustochytrids). Public Output report of the ALG-AD project, Swansea, November 2020, 17 pages.

Available online at www.nweurope.eu/projects/ALG-AD.

This document is an output from the ALG-AD project, which has received European Regional Development Funding through the INTERREG IVB NWE programme.

UNDEB EWROPEAIDD EUROPEAN UNION
Llywodraeth Cymru Welsh Government
Cronfa Datblygu Rhanbarthol Ewrop
European Regional Development Fund

Executive summary

This document is a technical report detailing the methods required to extract oils and peptides from Thraustochytrids. This document is part of the INTERREG North West Europe funded ALG-AD project aiming to combine anaerobic digestion (AD) and microalgal cultivation technologies to remediate nutrient-rich digestate currently produced in excess in the area.

The main objective of this report is to show how this scientific task was carried out in the laboratory and at the pilot facilities based at industrial locations. The document draws conclusions and recommendations from laboratory and large-scale trials conducted at the ALG-AD project investment site in France.

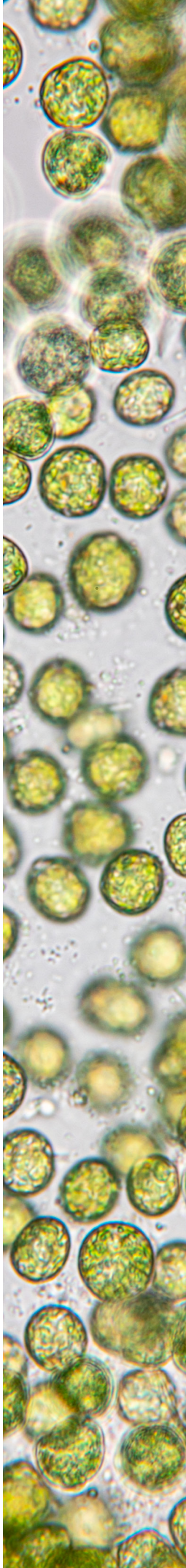


TABLE OF CONTENTS

0 INTRODUCTION

Thraustochytrids are osmo-heterotrophic protists present in benthic food webs. Although their ecology and physiology are still very poorly known, they are widely distributed in marine habitats (from coastal to deep seas) characterized by the accumulation of large quantities of refractory organic matter. Even if their 'numerical' abundance is low compared to prokaryotes, their role in the degradation of various organic substrates is a determining factor in the functioning of marine microbial loops (Raghukumar, 2002).

These marine protists are single cell organisms with a non-cellulosic cell wall which consist of sulphated polysaccharides enriched in galactose and xylose. They can also produce an ectoplasmic net (a rhizoid-like structure) which contains hydrolytic enzymes that allow penetration of solid surfaces (like algae or marine detritus) and breakdown of organic compounds for adsorption and transfer of nutrients. However, this structure can be more or less developed depending on the environmental conditions, but they generally do not produce it when grown in rich liquid media (Raghukumar, 2002).

They have received considerable attention for their capacity to produce n-3 polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (C22:6n-3, DHA). It is known that these PUFA are essential for animal and human health. They play important roles in the regulation and protection of the cardiovascular and nervous systems, and are beneficial for their anti-inflammatory effects, and their protective function against neurodegenerative diseases. (Nicolson and Ash, 2017).

However, numerous studies have revealed the presence of other interesting compounds in thraustochytrids such as molecules with antimicrobial activity (Hang et al, 2019), astaxanthin (Quilodran et al, 2010) or squalene (Jiang et al, 2004).

Bioactive peptides produced through enzymatic hydrolysis of proteins can also increase the nutritional value of thraustochytrids biomass (Cai et al, 2017). So far few works have employed enzymatic hydrolysis on thraustochytrids biomass. Lowry et al (2016) used Alcalase to hydrolyse the spent media of a thraustochytrids culture with the aim of reusing it as a culture medium. Other authors used this process to enable lipid extraction (Byreddy, Barrow and Puri, 2016; Lin et al, 2018) or to produce protein hydrolysates after lipid extraction (Lin et al, 2015; Wang et al, 2018).

In the process of enzymatic hydrolysis, it is important to be able to establish how the degree of hydrolysis (DH) of the biomass is evolving. The pH-Stat technique allows us to monitor the DH continuously and at real-time by means of proton titration. During the hydrolysis of a protein, molecules with a carboxyl group and molecules with an amine group are released. At neutral or basic pH, the carboxyl groups release protons which are titrated with the addition of NaOH. But at the same time, the amine groups consume protons to form ammonium groups. So, in order to convert the volume of NaOH added in DH it is necessary to take into account the degree of dissociation of the amine groups (α) which will depend on the pH and temperature of the reaction.

The ALG-AD project aims to optimize enzymatic proteolysis of *Aurantiochytrium mangrovei* (thraustochytrid) grown on agricultural effluents in order to obtain a food supplement rich in polyunsaturated fatty acids (PUFAs) and peptides. In this work, the action of the Alcalase was studied using the pH-Stat technique. Unlike other works, the biomass will be hydrolyzed in its whole, i.e. without a previous lipid separation.

1 MATERIALS AND METHODS

Biomass production

Although the objective is to have a biomass of the same quality between the different productions, some factors (seasonal changes of temperature, filtration conditions, etc) are not yet fully controlled. These factors could potentially influence the biochemical composition of the biomass or its percentage of dry matter.

Here we worked with the biomass from three batches produced at different records (Table 1) to assess the differences between their hydrolysis products.

Biomass ID	Date of Production
P20D	January 2020
P20F	June 2020
P20I	July 2020

Table 1: Date of Production of the batches used in this study

Culture conditions

The production of biomass was carried out in 500L cylindrical tanks. The culture medium is detailed in Table 2.

Culture medium in pilot production	
MGSO4	0.5 g/L
Yeast Extract	2 g/L
Peptone	2g/L
Salt	15g/L
Glucose	20g/L
Digestate	2.5 g/L

Table 2 A. mangrovei culture media composition

The digestate was obtained from Cooperl, an animal feed mill and slaughterhouse cooperative located in Lamballe (France). Digestate is the product of the methanisation process in which all liquid and semi-solid residues from the plant are treated (pig manure, wastewater from the slaughter house, muds from the denitral slurry station, etc). Before use, the digestate was filtered through a 300 kDa membrane. After filtration, the digestate had 2.23 g/L of N and 0.04 g/L of P.

The targeted temperature during the culture was set at 28 °C but it varied throughout the day and between productions (which were done in different seasons) from 23°C to 32°C. The production of foam was regulated with the addition of silicone antifoam and the pH with the addition of NaOH 10 N. Aeration was set at 200 L/min. The nomenclature of culture batches was P for production, 20 for 2020 and a letter for each week batch (from D to M).

After 28h for P20D and P20F and 24h for P20I the culture was harvested and concentrated through tangential filtration. The biomass was aliquoted into 400 mL and stored at -20°C until hydrolysis

Biomass filtration

Culture condition variations during production can result in cellular morphological and physiological variations which may influence their resistance to tangential filtration. To minimize the loss of biomass, different filtration conditions were evaluated to preserve biomass quality at last.

In the case of P20D, the filtration was carried out by the company Polymaris, which uses a tangential filtration system on a 300 kDa membrane of 14.8 m^2 first, and on a 300 kDa membrane of 0.2 m^2 in a second time. After the biochemical and microscopic analysis of the biomass, a significant loss of biomass was observed due to the rupture of the cells during filtration.

The following cultures were filtered at the Cooperl site with a tangential filtration pilote on a 300 kDa membrane (14 membranes with 11 canals, total surface 3.5 m^2). The rate of filtration was modified

5

between the production of the P20F and P20I biomasses to limit cell disruption.

Monitoring of enzymatic hydrolysis with the pH-Stat method

The enzyme chosen to hydrolyze the thraustochytrids biomass was Alcalase 2.4L (Sigma-Aldrich, a protease of *Bacillus licheniformis*). It has an optimal pH of 7 - 8, a density of 1.25 g/mL and its declared proteolytic activity is 2.4 Anson units/g (One unit is defined as the amount of enzyme that will release 1.0 µmol L-tyrosine from hemoglobin per min at 25°C, pH 7.5).

The degree of hydrolysis (DH) was determined using the pH-Stat method in which a base (NaOH 0.5 N) is added to keep the pH constant throughout the reaction. DH can be calculated from the sodium hydroxide titration of the amino groups liberated during hydrolysis in alkaline medium, according to the following equation (Adler Nissen, Eriksen and Olsen, 1983):

$$DH\ (\%) = \frac{B\ x\ N_b}{\alpha\ x\ Mp\ x\ h_{tot}}\ x\ 100$$

Where B is the volume of titrant (mL), N its normality (meq/mL), α is the mean degree of dissociation of α-amino groups, Mp is the protein mass (g) and htot is the number of peptide bonds per gram of proteins. In this work the α values calculated by Adler Nissen, Eriksen and Olsen (1983) were used. For the hydrolysis carried out at pH 7, α was 0.44 and for those carried out at pH 8 it was 0.88. The value for h_{tot} was calculated from the characterization of the total amino acid profile determined by performic oxidation (UPSCIENCE Labs) of P20F biomass. The same h_{tot} value was used for the P20D and P20I biomasses.

Hydrolysis conditions

The hydrolysis were carried out in 500 ml and 300 ml reactors, heated with a water bath at 50°C and stirred at 500 rpm with a 3-bladed propeller. The pH was adjusted before enzyme addition and regulated during the hydrolysis with NaOH.

More details about each experience are given below.

Experiments with P20D biomass

All the experiments were performed in a 500 mL double walled reactor. Temperature was set at 50°C and the biomass was stirred at 500 rpm with a 3-bladed propeller. The pH was adjusted before enzyme addition and regulated during the hydrolysis with NaOH 0.5N.

In order to obtain a maximum of information with a small number of experiences, a two-factor factorial experience plan was carried out. The response measured was the degree of hydrolysis (DH) calculated

Total amino acids	g/100g	meqv/g of protein
aspartic acid	3.17	0.69
threonin	1.59	0.39
serine	1.44	0.40
glutamic acid	3.9	0.76
proline	1.42	0.36
glycine	1.5	0.58
alanine	2.23	0.72
cystine	0.36	0.04
valine	1.85	0.46
methionine	0.61	0.12
isoleucine	1.5	0.33
leucine	2.34	0.51
tyrosine	1.11	0.18
phenylalanine	1.29	0.23
histidine	0.59	0.11
lysine	2.06	0.41
arginine	1.24	0.21
hydroxyproline	<0.20	0.00
hydroxylysine	0.02	0.00
Total	28.22	6.47

Table 3 Amino acid composition of A. mangrovei biomass on p20f batch

with the pH-Stat method and the factors studied were the pH (7 and 8) and the percentage of enzyme calculated on the basis of the amount of protein present in the reactor (1.9% and 3.8% w/w) (Table 4).

With the results obtained, it was possible to evaluate the effect of each factor and the type of interaction (positive or negative) between both of them. The equations used to analyze the data are detailed in Table 5:

In addition, hydrolysis without addition of Alcalase at pH 7 was performed to evaluate the autolysis by endogenous enzymes in the biomass.

N° of assay	Enzyme	pH	Result (DH)
1	1.9%	7	Y1
2	3.8%	7	Y2
3	1.9%	8	Y3
4	3.8%	8	Y4

Table 4 Experimental design for the study of pH and enzyme concentration on p20D biomass

Media	$\hat{a}_0 = \frac{1}{4}[+y_1 + y_2 + y_3 + y_4]$
Effect of 1	$\hat{a}_1 = \frac{1}{4}[-y_1 + y_2 - y_3 + y_4]$
Effect of 2	$\hat{a}_2 = \frac{1}{4}[-y_1 - y_2 + y_3 + y_4]$
Interaction	$\hat{a}_{12} = \frac{1}{4}[+y_1 - y_2 - y_3 + y_4]$

Table 5 Equations used to determine the effect of pH and enzyme concentration on the dh of p20D biomass

Experiments with P20F biomass

The hydrolysis of P20F biomass were carried out at pH 7 in a 300mL reactor heated with a water bath at 50°C and stirred at 500 rpm with a 3-bladed propeller. The amounts of enzyme evaluated were 4.3% and 2.2% (w/w) and a treatment without enzyme was also carried out at pH 7. The pH was adjusted before enzyme addition and regulated during the hydrolysis with NaOH 0.5N

Experiments with P20I biomass

The first hydrolysis of this biomass was carried out at pH 7 with 7.3% of enzyme (w/w) in a 300 mL reactor heated with a water bath at 50°C and stirred at 500 rpm with a 3-bladed propeller. For this biomass, the pH was adjusted before enzyme addition with NaOH 2N and regulated during the hydrolysis with NaOH 0.5N.

Another hydrolysis was carried out at pH 7 with 4.4% enzyme in a 500 mL reactor. This time the pH was not regulated during enzyme action but conductivity was monitored.

Biochemical characterization of hydrolysates

In order to quantify the enzyme efficiency for biomass hydrolysis, samples were taken during hydrolysis to i) analyze the peptide and lipid profiles of the biomass and ii) make observations by optical microscopy. All samples are treated at 80°C for 20 minutes in a water bath to inactivate the enzyme. They are then stored at -20°C until biochemical analysis.

Peptide analysis by Size Exclusion HPLC (SEC-HPLC)

The hydrolysates were filtered on PVDF filters with a low protein absorption rate (Millex®, 33mm, 22µm) then analyzed by SEC-HPLC on a Superdex Peptide 10/300 GL column with a fractionation range of 100 to 7000 Da (10x300-310nm, GE Healthcare Bio-sciences).

The mobile phase is a solution composed of 70% water + 0.1% trifluoroacetic acid and 30% acetonitrile. The flow rate is set at 0.5 mL.min-1 and the absorbance is measured at 214 nm.

A calibration curve was carried out with the following standards to know the molecular weight profile of the peptides present in the hydrolysate (Figure 1): Glycine (75 Da), L-threonine (119 Da), L-asparagine (132 Da), Leupeptin (463 Da), Substance P F1-7 (900 Da), Pituitary Gonadotropin Releasing Hormone (LH-RH) (1182

Da), Substance P acetate (1347 Da), Neurotensin (1672 Da) and Insulin (3495 Da).

Although it is not possible to establish the concentration of each compound with this method, to evaluate the success of the hydrolysis it is possible to observe the change in absorbance at equivalent retention rates.

In order to analyze the chromatograms, 7 regions based on retention times were defined to analyze the area of the compounds produced during the hydrolysis (Figure 2). The same retention times were kept for all the chromatograms with minor adjustments of some seconds for some samples (Table 6).

Analysis of lipid content and fatty acid composition

Total lipids were extracted by addition of 6 mL of chloroform: methanol (2:1) to the freeze-dried sample. The mixture was then exposed to ultrasound for 10 minutes and finally it was agitated at 600 rpm during 30 minutes.

Polar and neutral fatty acid content

For the fatty acid (FA) analysis in polar and neutral lipids, an aliquot of the chloroform: methanol (2:1) extract was dried under N_2 and then resuspended in chloroform: methanol (98:2, v:v). Separation of neutral and polar lipids was then realized by solid phase extraction (Le Grand et al. 2014). Each fraction (polar lipids and neutral lipids) was transesterified and the resulting fatty acid methyl esters (FAME) were analyzed and quantified by gas chromatography (GC-FID) according to the method from Le Grand et al. (2014). Lipid class and fatty acid standards were those used in Le Grand et al. (2014).

Compound	Retention Time
A (15-6 KDa)	10-20 min
B (6-0.6 KDa)	20-34 min
C (0.6-0.3 KDa)	34-39 min
D (0.3-0.2 KDa)	39-43 min
E (<0.1 KDa)	43-46 min
F (<0.1 KDa)	46-49 min
G (<0.1 KDa)	49-60 min

Table 6 Retention times of the compounds found on the peptide analysis of *A. mangrovei* biomass

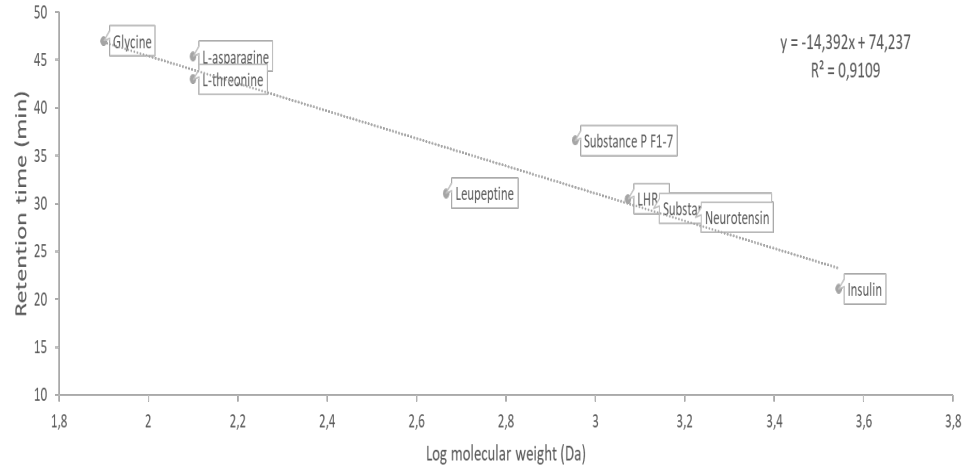


Figure 1 Calibration curve for SEC-HPLC analysis

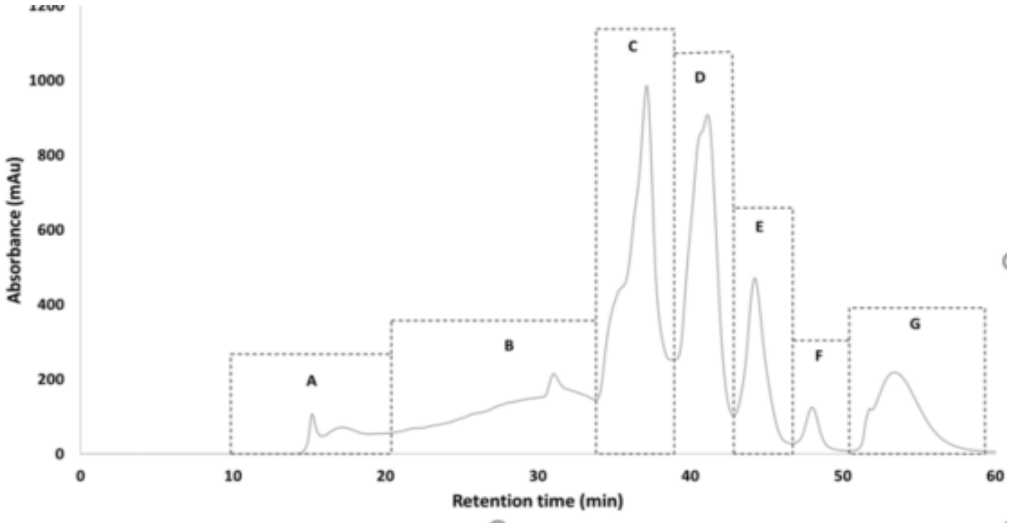


Figure 2 example of chromatogram obtained during SEC-HPLC analysis

2 RESULTS

Alcalase-free treatment

Alcalase-free hydrolysis was performed as a control to evaluate the autolysis due to endogenous enzymes of thraustochytrids. During this treatment, the biomass was heated at 50°C and agitated at 500 rpm during two hours.

At T0, when the enzyme has not yet been added to the biomass, the presence of peptides of different molecular weights can be observed on biomasses P20D and P20F (Figure 3). Filtration and freezing of the biomass are probably the main reason for the release of intracellular peptides prior to hydrolysis. According to the images taken before and after the filtration and freezing (Figure 4), it can be seen that this process produces the aggregation and rupture of many cells, which may release their intracellular content into the medium.

Although all hydrolysis were conducted with previously filtered and frozen biomass, it is likely that this is a favorable pre-treatment for hydrolysis as the intracellular proteins are directly available to be digested, reducing the hydrolysis time and the amount of enzyme to be used

In Figures 5 and 6 the percentage of area of the peptide compounds is shown for each biomass. Even if there is a slight variation of the area of some compounds between different sampling times, the area

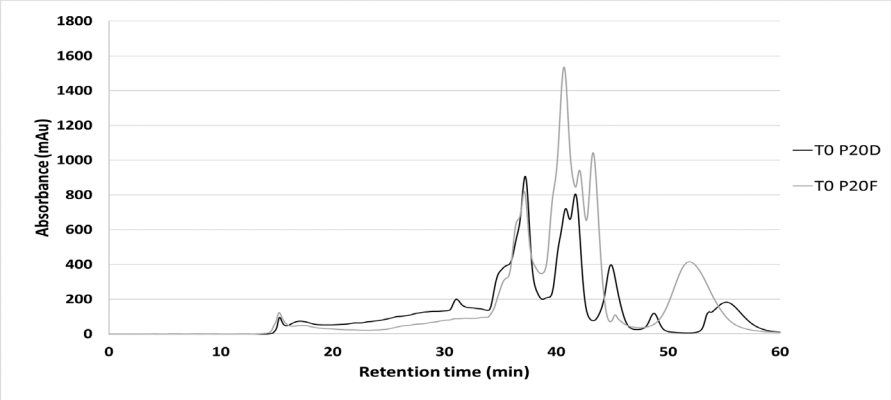


Figure 3 peptide profile of P20D and P20F biomasses before hydrolysis

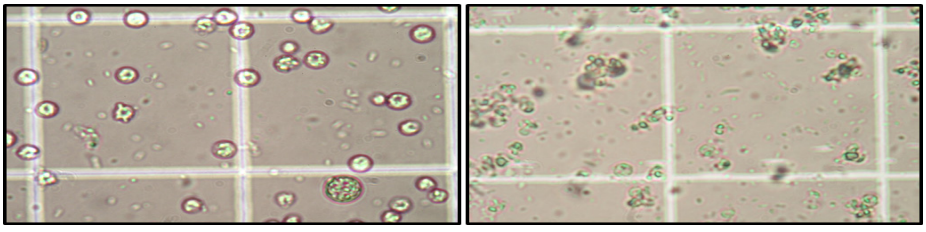


Figure 4 Cells before (left) and after (right) filtration and freezing

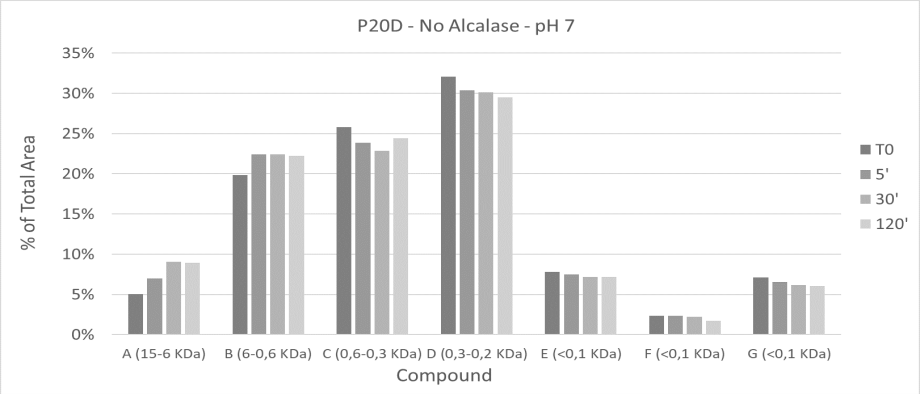


Figure 5 Percentage of total area of peptide compounds present in P20F biomass during treatment without enzyme at different sampling times

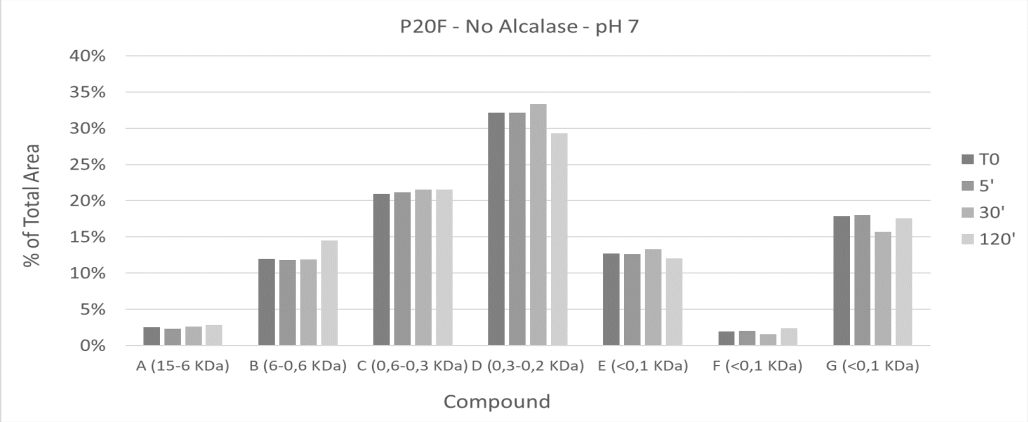


Figure 6 Percentage of total area of peptide compounds present in P20F biomass during treatment without enzyme at different sampling times

remains quite stable during the treatment without the addition of enzyme. Thus, we concluded that endogenous enzymes are little active.

If endogenous enzymes were active in the biomass, the process would be more complex as they would have to be deactivated. The activity of unknown enzymes in our biomass would not have made possible a correct control of the hydrolysis.

Note about pre-titration

In the case of P20D biomass, 43.8 mL of NaOH 0.5 M were added to reach the set point pH and this process lasted 35 min. After the pre-treatment and until the end of the two-hour treatment, 2.14 mL of NaOH 0.5M were added and a final DH of 2.28 was obtained.

For P20F biomass, the pretitration lasted 50 minutes and 43 mL of NaOH 0.5 M were added. Although a two-hour hydrolysis was intended, the base addition stopped after 33 minutes as the pH started to increase above 7. At the end of the two-hour treatment the pH reached a value of 7.11. In total 0.67 mL of NaOH 0.5M were added obtaining a DH of 0.81.

Treatment of P20D biomass with Alcalase 2.4 L

The DH of each experience is detailed in Table 7 and the results of the experience plan in Table 8.

The effect of the enzyme is of 1.03 for a variation of 0.95% enzyme and the pH effect is negative (-0.5) for a 0.5 unit pH variation.

Figures 7 to 10 show the effect of each treatment on the area (mAu.min) of the peptide compounds of the biomass. In all cases, when the enzyme is added there is a significant increase in the area of compounds B and C (compounds of 6 to 0.3 kDa). Only in the case of the treatment with 1.9% of enzyme at pH 8 the maximum area of all of the compounds is obtained at the end of the hydrolysis (120 min). However in the rest of the treatments the hydrolysis seems to reach its maximum between 5 and 30 minutes. It is interesting to note that the area of compound B begins to decrease after it has reached its maximum. Probably the enzyme starts to digest this type of compound when there is no other substrate left.

These results permitted an initial characterization of

N° of assay	Enzyme	pH	Result (DH)
1	1.9%	7	Y1: 11.71
2	3.8%	7	Y2: 14.26
3	1.9%	8	Y3: 11.18
4	3.8%	8	Y4: 12.77

Table 7 experimental design to evaluate the effect of pH and enzyme concentration

Media	12.48
Effect of 1	1.03
Effect of 2	-0.5
Interaction	-0.24

Table 8 Effects of the factors pH and enzyme concentration

the biomass and the determination of the hydrolysis conditions that would be practiced on the other batches. Considering these results it can be established that although DH can be increased with the addition of more enzyme, this can lead to a greater digestion of the first products of its action between 6 and 0.3 kDa.

The pH seems to have little influence on the hydrolysis conditions performed. Although it is necessary to carry out repetitions to determine whether these effects are significant or not, it is more practical and economical to work at pH 7 given that the biomass of thraustochytrids is a very acidic biomass. If the effect of pH 8 is not significant on the hydrolysis yield, then it is preferable to work at pH 7.

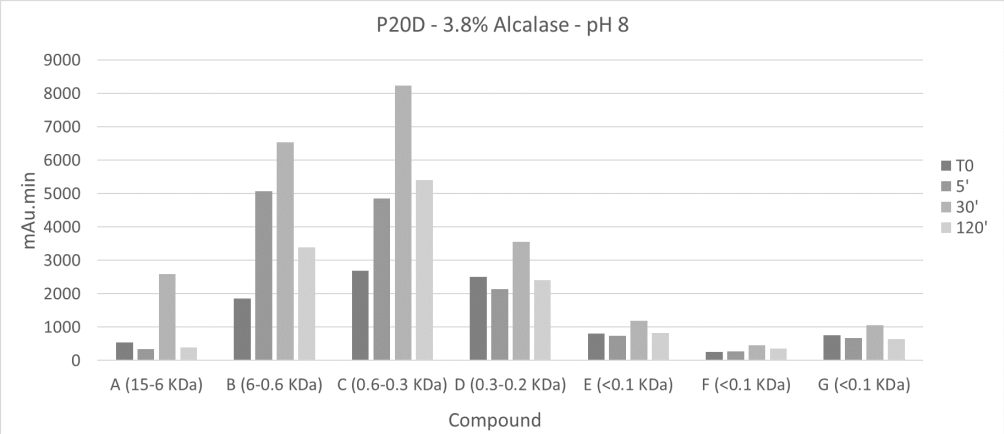


Figure 7 Peptide profile of P20D biomass with 1.9% Alcalase at pH 7

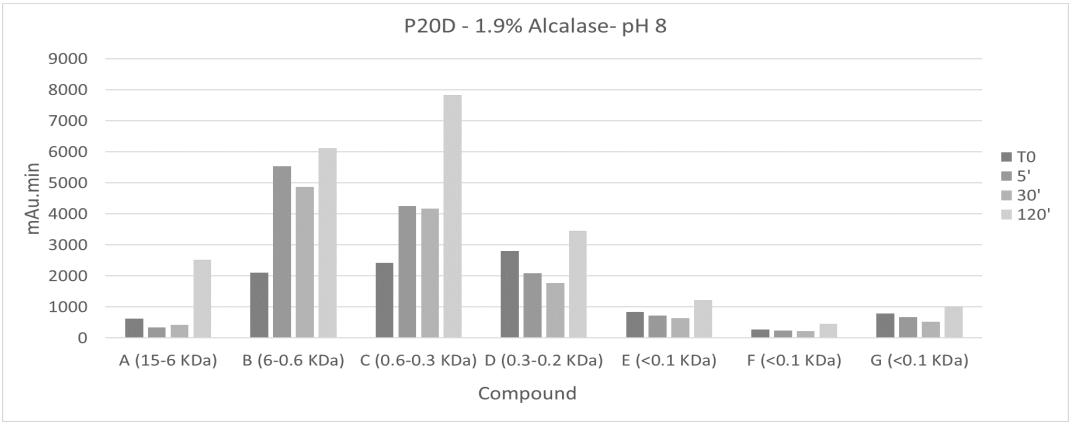


Figure 8 Peptide profile of P20D biomass with 1.9% Alcalase at pH 8

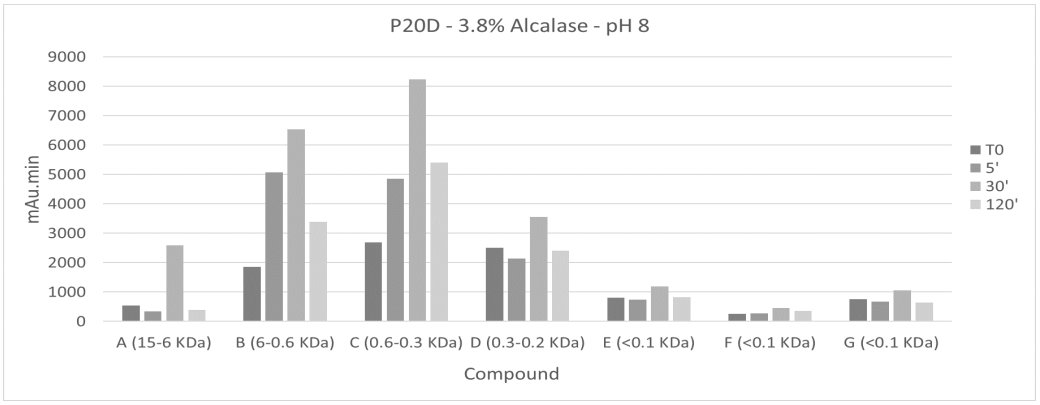


Figure 9 Peptide profile of P20D biomass with 3.8% Alcalase at pH 7

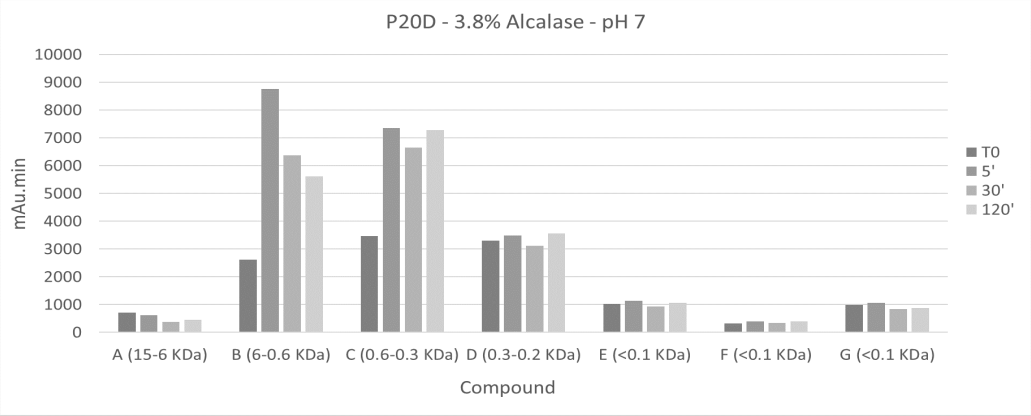


Figure 10 Peptide profile of P20D biomass with 3.8% Alcalase at pH 8

Treatment of P20F biomass

This biomass was treated with 2.2% and 4.3% of Alcalase at pH 7. In the case of the treatment with 2.2% of Alcalase a DH of 3.5 was obtained. Compounds B and C increased by 5 and 2.7 times respectively in 17 minutes and from that moment and up to the end of the hydrolysis the area of both compounds decreased (Figure 11).

As for the treatment with 4.3% of Alcalase, the DH was of 6 and the area of compounds B and C increased by 3.5 and 2 times respectively in the first 5 minutes of hydrolysis (Figure 12).

Similarly to the treatment without enzyme, NaOH addition stops completely before the 120 minutes of hydrolysis because the pH began to increase above the pH set point. In the case of treatment with 2.2% of Alcalase, pH increase begins after 42 minutes and a final pH of 7.10 was reached, while the pH of the treatment with 4.3% of Alcalase began to increase approximately at 75 minutes until a value of 7.05 at 120 minutes.

At the end of the hydrolysis, the hydrolysate was centrifuged at 15000g for 20 minutes (Figure 13). It can be seen that the biomass accumulated in the bottom decreased with the enzyme treatment. On the contrary, the top layer remained after hydrolysis even though its appearance changed drastically. The bio-

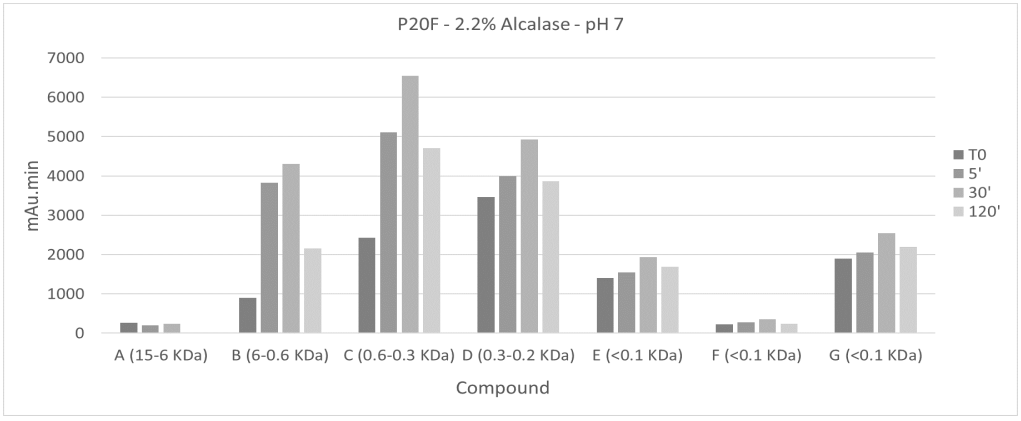


Figure 11 Peptide profile of P20F biomass with 2.2% Alcalase at pH 7

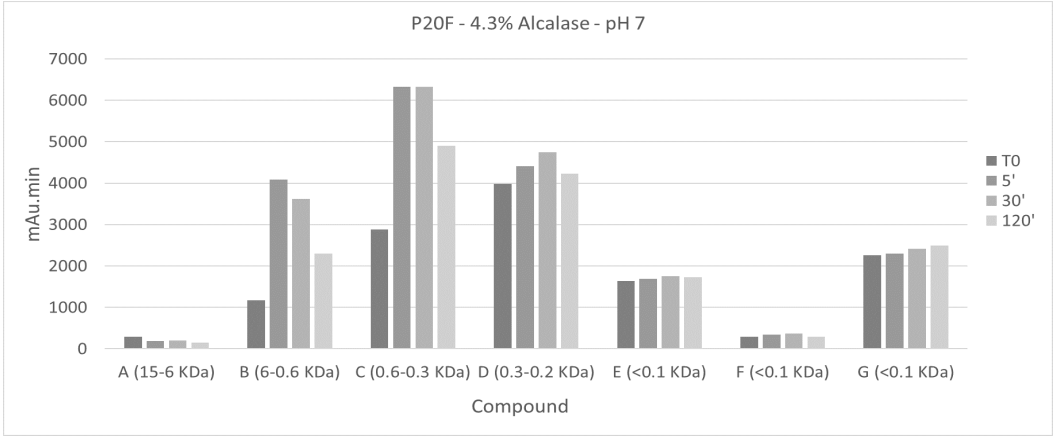


Figure 12 Peptide profile of P20F biomass with 4.3% Alcalase at pH 7

mass on the top layer looks like an emulsion. According to a study made on fava bean protein isolate, the hydrolysis with Alcalase can improve adsorption at the octanol/water interface and the emulsifying capability (Liu et al 2019)

Treatment of P20I biomass

A DH of 6 was obtained during the treatment with 7.3% Alcalase and an increase of 2.6 fold of compound B was obtained in 5 minutes of hydrolysis of P20I biomass (Figure 14). However, with such a high amount of enzyme, hydrolysis occurred much faster and that within 5 minutes of treatment the products of the enzyme such as compound B are partly digested.

When the hydrolysis was performed without pH adjustment, the areas of compounds B and C also increased with the addition of the enzyme. Even though enzyme concentration was lower, it did not impact the amount of peptide released (Figure 15). The change in pH and conductivity during hydrolysis can be seen in

Figure 13 Hydrolysate of P20F biomass after centrifugation

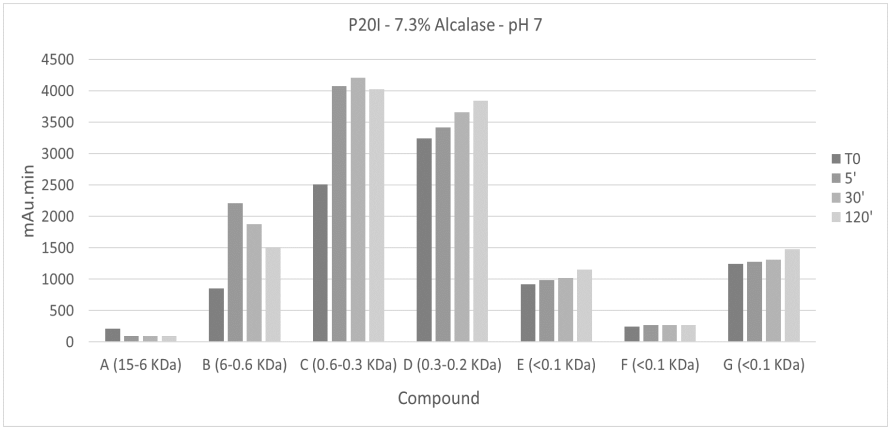


Figure 14 Peptide profile of p20I biomass with 7.3% Alcalase at pH 7

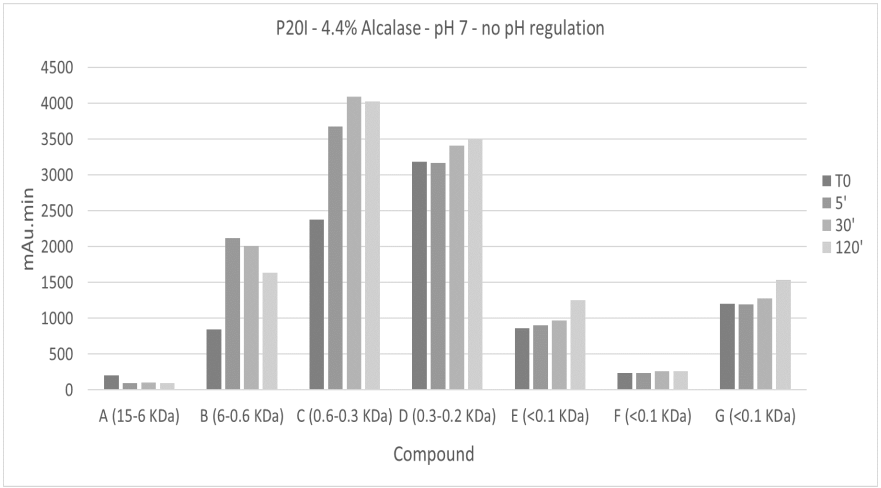


Figure 15 Peptide profile of P20I biomass with 4.4% Alcalase with no pH regulation

Figure 16.

Biomass digestion can be observed in Figure 17 and as in the case of P20F biomass, a top layer is formed during the hydrolysis.

Fatty acid analysis

Fatty acid analysis was carried-out to monitor the composition of the biomass after hydrolysis since the conditions during hydrolysis and inactivation of enzyme may degrade certain lipids.

As seen in Figure 18, fatty acid profile was quite stable between the non-hydrolyzed biomass (T0) and the hydrolyzed biomass (T7200).

The fatty acid analysis of the other batches and hydrolysis conditions is being made in parallel with an analysis to determine the peroxidation index of the biomass. This information will allow us to determine how the hydrolysis conditions affect the quality of the biomass

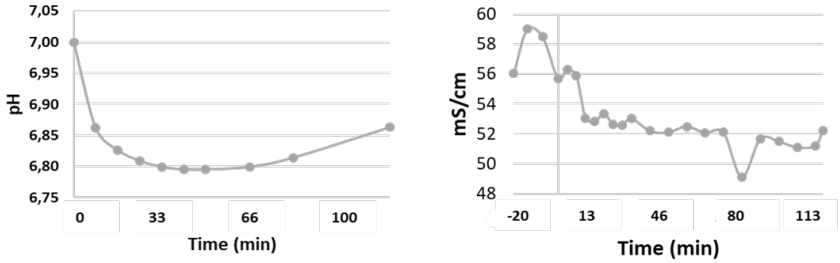


Figure 16 pH and conductivity variation during hydrolysis of P20I biomass without pH regulation

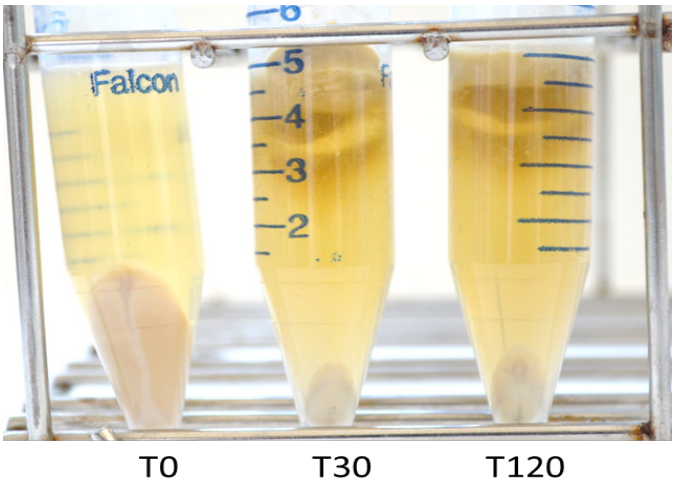


Figure 17 Hydrolysate of P20I biomass after centrifugation

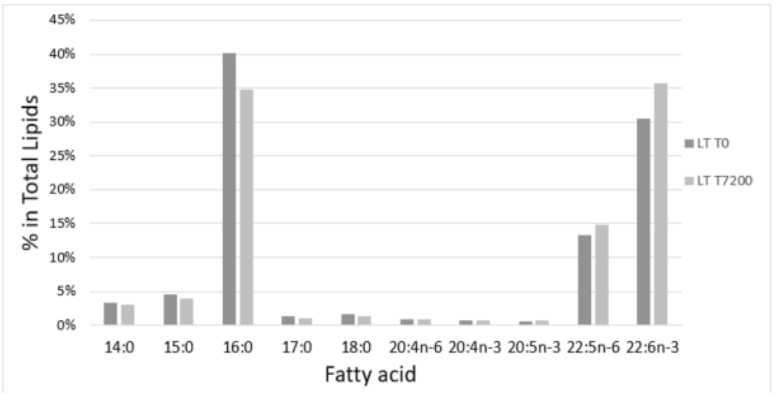


Figure 18 Percentage of different fatty acids in total lipids of P20F biomass hydrolyzed with 2.2% alcalase

3 CONCLUSIONS

This study allowed us to evaluate the action of Alcalase enzyme on *Aurantiochytrium mangrovei* biomass produced in pilot conditions.

From the comparison of the peptide profile at T0 and T5 min between the three batches (Figure 19-21), we can see that the peptide profile of the P20D biomass differed slightly from the other two batches (P20F and P20I) which were similar.

The three biomasses present low molecular weight peptides prior to hydrolysis. It is possible that the freezing of the biomass produces the rupture of the cells and the liberation of peptides and proteins to the medium. This facilitates hydrolysis since the proteins are already available to be digested.

Also, the endogenous enzymes do not seem to be active during hydrolysis which is an advantage since we do not need to do a pre-inactivation treatment.

On the hydrolysis carried out with enzyme, it mainly produced molecules between 6 and 0.3 kDa. In general, smaller compounds (<0.2 kDa) are not or only slightly affected by the conditions of hydrolysis.

According to the results obtained through the experience plan, it is possible to increase the DH with a higher concentration of enzyme (more than 4% E/S). However the compounds produced by the Alcalase reached their maximum area before the end of the

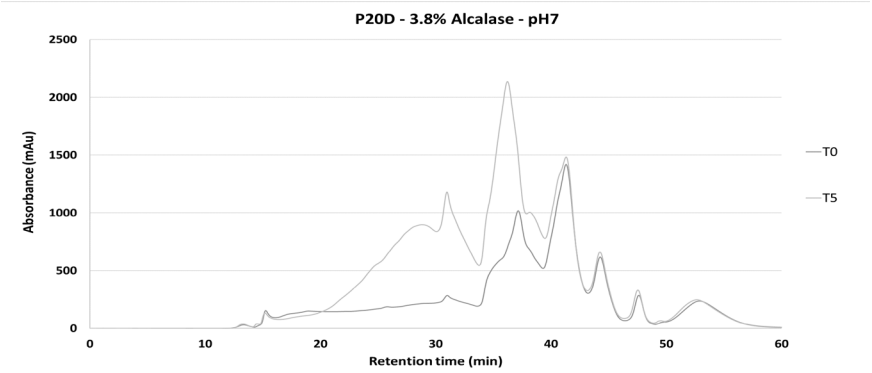


Figure 19 Chromatogram of the peptide fraction of P20D at T0 and T5 min

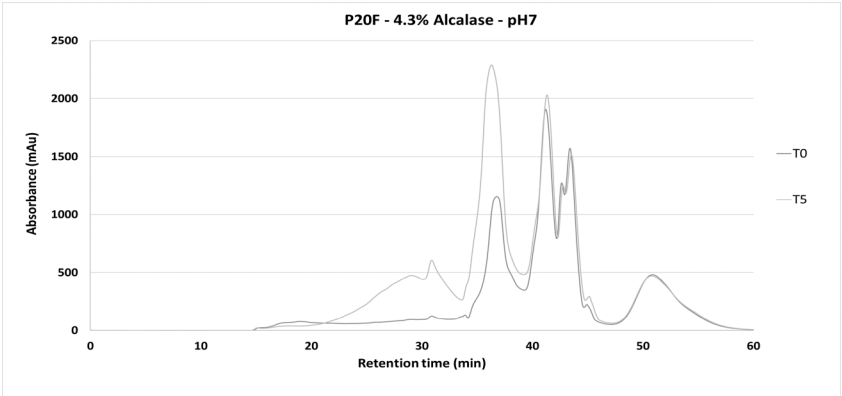


Figure 20 Chromatogram of the peptide fraction of P20F at T0 and T5 min

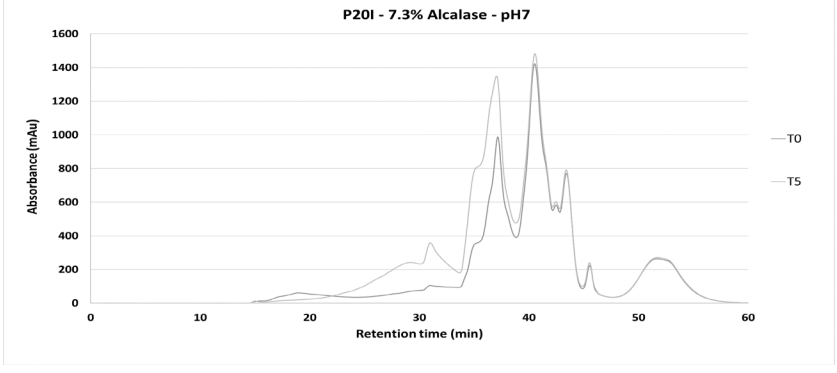


Figure 21 Chromatogram of the peptide fraction of P20I at T0 and T5 min

hydrolysis process, and then they started to decrease. It is likely that these compounds began to be digested to produce smaller size molecules.

Moreover, pH 8 do not seem to improve hydrolysis. Nevertheless, the lack of repetitions limited the statistical value of the experiences. Another experience with P20F biomass was carried out with 4% of enzyme at pH 7 and 8 to have more evidence about pH effect. The results are being currently treated.

When working with P20F biomass a higher DH is obtained with 4% of enzyme but the treatment with 2% of enzyme seemed more adequate since it provided a higher increase of compounds B and C at 17 minutes of hydrolysis.

Finally, it seems possible to perform the hydrolysis without pH adjustment, provided that the pH is adjusted prior to the addition of the enzyme because the pH of the biomass is too acid for the Alcalase activation.

The analysis of all the lipid samples taken during the experiments is in progress, but the results obtained so far suggest that the fatty acid profile of the biomass is not affected by hydrolysis.

REFERENCES

- Adler-Nissen, J., Eriksen, S. & Olsen, H.S. (1983). Improvement of the functionality of vegetable proteins by controlled enzymatic hydrolysis. Plant Foods for Human Nutrition 32, 411-423.

- Byreddy, A.R. ; Barrow, C.J. ; Puri, M. (2016). Bead milling for lipid recovery from thraustochytrid cells and selective hydrolysis of Schizochytrium DT3 oil using lipase. Bioresource Technology, 200, 464-469.

- Cai, X.; Yan, A.; Fu, N.; Wang, S. (2017). In Vitro Antioxidant Activities of Enzymatic Hydrolysate from Schizochytrium sp. and Its Hepatoprotective Effects on Acute Alcohol-Induced Liver Injury In Vivo. Marine Drugs, 15 (4), 115.

- Hang, N.T.M.; Giap, T.H.; Thanh, L.N.; Oanh, N.T.T.; Hong, D.D.; Minh, C.V. (2019). Metabolites from Microalgae Schizochytrium mangrovei. Chemistry of Natural Compounds, 55, 978-981.

- Jiang, Y., Fan, K. W., Wong, R. T. Y., & Chen, F. (2004). Fatty Acid Composition and Squalene Content of the Marine Microalga Schizochytrium mangrovei. Journal of Agricultural and Food Chemistry, 52 (5), 1196–1200.

- Le Grand, F.; Soudant, P.; Siah, A.; Tremblay, R.; Marty, Y.; Kraffe, E. (2014) Disseminated Neoplasia in the Soft-Shell Clam Mya arenaria: Membrane Lipid Composition and Functional Parameters of Circulating Cells. Lipids, 49, 807-818.

- Lin, J.; Cai, X.; Tang, M.; Wang, S. (2015). Preparation and Evaluation of the Chelating Nanocomposite Fabricated with Marine Algae Schizochytrium sp. Protein Hydrolysate and Calcium. Journal of Agricultural and Food Chemistry, 63 (44), 9704-9714.

- Lin, Y.; Xie, X.; Yuan, B.; Fu, J.; Liu, L.; Tian, H.; Chen, T.; He, D. (2018). Optimization of Enzymatic Cell Disruption for Improving Lipid Extraction from Schizochytrium sp. through Response Surface Methodology. Journal of Oleo Science, 67 (2), 215-224.

- Liu, C.; Bhattarai, M.; Mikkonen, K. S.; & Heinonen, M. (2019) Effects of Enzymatic Hydrolysis of Fava Bean Protein Isolate by Alcalase on the Physical and Oxidative Stability of Oil-in-Water Emulsions. Journal of Agricultural and Food Chemistry, 67 (23), 6625-6632.

- Lowrey, J., Brooks, M.S. & Armenta, R.E. (2016). Nutrient recycling of lipid-extracted waste in the production of an oleaginous thraustochytrid. Applied Microbiology and Biotechnolgy,100, 4711-4721.

- Nicolson, G.L.; Ash, M.E. (2017). Membrane Lipid Replacement for chronic illnesses, aging and cancer using oral glycerolphospholipid formulations with fructooligosaccharides to restore phospholipid function in cellular membranes, organelles, cells and tissues. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1859, 1704-1724.

- Quilodrán, B., Hinzpeter, I., Hormazabal, E., Quiroz, A., & Shene, C. (2010). Docosahexaenoic acid (C22:6n-3, DHA) and astaxanthin production by Thraustochytriidae sp. AS4-A1 a native strain with high similitude to Ulkenia sp.: Evaluation of liquid residues from food industry as nutrient sources. Enzyme and Microbial Technology, 47, 24-30.

- Raghukumar, S. (2002). Ecology of the marine protists, the Labyrinthulomycetes (Thraustochytrids and Labyrinthulids). European Journal of Protistology, 38, 127-145.

- Wang, X.; Wang, H.; Pierre, J.F.; Wang, S.; Huang H.; Zhang, J.; Liang S.; Zeng, Q.; Shang, C.; Huang, M.; Ruan, C.; Lin, J.; Li, H. (2018). Marine microalgae bioengineered Schizochytrium sp. meal hydrolysates inhibits acute inflammation. Scientific Reports, 8.