Waste and Biomass Valorization
https://doi.org/10.1007/s12649-020-01029-x

ORIGINAL PAPER

Enzymatic Hydrolysis of Fish Waste as an Alternative to Produce High Value-Added Products

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Received: 24 October 2019 / Accepted: 16 March 2020

Abstract

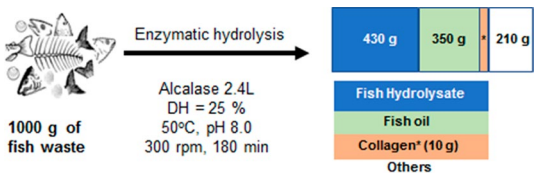
Purpose Fish waste was studied as a raw material for the simultaneous production of protein hydrolysates, collagen and fish oil. Enzymatic hydrolysis was selected for recovering these by-products with high value-added.

Methods Alcalase 2.4 L was used to hydrolyze fish waste in a batch reactor under controlled conditions (180 min, 50 °C and pH 8). The influence of hydrolysis degree on by-products recovery was analyzed for different enzyme and substrate concentrations.

Results Results suggested that the enzyme/substrate ratio was the main factor controlling the hydrolysis rate. Linear relationships were found between the degree of hydrolysis and the amount of each of the obtained by-products. From these relationships, the amounts of by-products with high added value can be predicted by only knowing the degree of hydrolysis reached. In optimal conditions (DH = 25%), 430 g of protein hydrolysate, 10 g of collagen and 350 g of oil could be obtained from 1000 g of fish waste. The use of fish waste as raw material for by-product fabrication resulted in a 79% reduction of waste disposed to landfill.

Conclusion Therefore, this study shows the enzymatic hydrolysis of fish waste as a feasible solution to obtain high value-added products and an alternative to landfilling disposal.

Graphic Abstract



Keywords Enzymatic hydrolysis · Alcalase · Fish protein hydrolysate · Fish collagen · Fish oil

Statement of Novelty

Global fish consumption has more than doubled in the past 50 years. As a consequence, a significant amount of fish waste (rich in nutrients) is discarded each year and disposed of in the landfill and in the ocean. Against this background, this study develops an innovative treatment for the transformation of fish waste in new products with higher added value and a large demand: protein hydrolysates, collagen and oil. The proposed treatment is an original recycling method using enzymatic hydrolysis that allows the *simultaneous production* of the desired products.

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Published online: 28 March 2020

Introduction

In the last decades, world fish consumption has been increasing every year. In the 1960s, the per capita consumption worldwide was about 9.0 kg. This amount nearly doubled by the year 2013, when it reached more than 20 kg per person [1]. The European Union also follows this trend. It is estimated that from 1998 to 2030 the per capita consumption will increase from 22 to 24 kg among its members; it is worth mentioning the countries of the Iberian Peninsula—Spain and Portugal—that are expected to consume 39 and 57 kg of fish per person in 2030, respectively [2].

The increasing of global fish consumption can be explained by many factors such as an increasing population, rising incomes and urbanization, plus the increasing number of fishing industries and new and more modern ways of distribution of the industrialized frozen fish around the world [1]. About 70% of the fish consumed worldwide is processed in the industry before being sold. After processed, from 20 to 80% of the weight of the fresh fish is sent to the market [3]; this value varies according to the level of processing and the kind of fish [4]. In the processing for white fish fillet production, for example, for each 1000 g of processed fish, from 490 to 640 g are discarded: 240–340 g of bones and viscera, 210–250 g of heads and the rest are skins [5]. Some of these residues are used as ingredients in the production of animal feed [6] but, most of the fish processing waste is dumped in the sea and landfills [7]. However, fish waste has a protein concentration very similar to the parts of fish used for human consumption. It may be destined for a nobler application since fish proteins are superior when compared to the proteins originate from plants, and have a better amino acids balance when compared to other sources of animal protein [8].

Protein hydrolysates providing mainly di- and tripeptides are superior to intact (whole) proteins and free amino acids to be applied in several areas, such as nutrition [9, 10], biotechnology [11, 12] and cosmetics industries [13]. Therefore, the production of protein hydrolysates is an option to generate more income for the fish processing plant. The enzymatic hydrolysis has several characteristics that can facilitate its adoption in the treatment of fish waste: it is considered a quick and easily reproducible method; it can separate not only the peptide fractions but also oils from the insoluble solids; prevents extreme physical and chemical treatments; compared to chemical hydrolysis, it has the advantage of avoiding generating chemical waste, besides being more easily controlled, thus reducing unwanted reactions that destroy high-value components of the proteins [14]. However, a disadvantage of enzymatic hydrolysis could be the high price of some enzymes. For these reasons, the enzymatic hydrolysis is an alternative for the recovery of proteins and generating products with the potential to be applied in the previously cited areas. The wide variety of applications of hydrolyzed protein from fish occurs mainly due to their excellent balance of amino acids, good digestibility, and its rapid uptake, as well as good functional properties such as high solubility in water [15, 16].

Collagen is one of the main proteins present in fish waste. It is an insoluble fibrous protein, and its hydrolysis is more difficult than for globular proteins existing in these wastes. The low collagen hydrolysis is advantageous because it allows the recovery of the collagen for commercial uses: biomedical applications, cosmetics manufacturers, etc.[17, 18]. Currently, most of the collagen is extracted from bovine and porcine bones and skins; however, it is not acceptable by Hinduism, Islam, and Judaism because of religious restrictions [19]. In addition, collagen derived from prion-contaminated bovine supplies might be a potential source of exposure to bovine spongiform encephalopathy [20]. Therefore, fish waste is an interesting source for collagen production in the substitution of mammalian sources.

As previously indicated, fish oil can also be separated by enzymatic hydrolysis [21, 22]. The demand for fish oil has been increasing in the recent past due to the findings of the health benefits provided by omega-3 fatty acids. It is applied in cosmetic, pharmaceutical [23] and biodiesel industries [24].

Despite the growing demand for these three products (protein hydrolysates, collagen, and fish oil), to the best of our knowledge, no studies have been carried out to use the fish waste as a source for the *simultaneous production* of them. Against this background, this study aimed to determine the efficiency of the simultaneous production of protein hydrolysates and recovery of collagen and oil from the fish waste using proteases as catalysts for the hydrolysis. Enzyme concentration, fish waste concentration and enzyme/substrate ratio were analyzed to establish a relationship between the degree of hydrolysis and the production of by-products allowing to predict easily the amounts of products that can be obtained under any operating conditions.

Materials and Methods

Materials

Fish waste was collected from a local fish store. The residue was predominantly composed of medium-sized fish heads (like salmon), skins, viscera, mangled muscles of fish, small fishes, as well as mollusks such as squid and mussels. Fish waste was transported to the laboratory, ground

to size ≤ 1 mm, homogenized and frozen in small portions at – 20 °C. Prior to the hydrolysis process, a portion of the frozen waste was thawed overnight in a refrigerator at 4 °C.

In order to select the best protease to hydrolyze the fish waste, the material was independently hydrolyzed in previous studies by Alcalase 2.4 L, Flavourzyme 1000 L, Neutrase 0.8 L, Pancreatic Trypsin 6.0 S and Protamex from Novozymes A/S (Bagsvaerd, Denmark), respectively. The Alcalase gave the highest degree of hydrolysis of all hydrolysates, which agrees with the results of other studies [25]. Therefore, Alcalase 2.4 L was employed in this research. This enzyme is an endopeptidase from *Bacillus licheniformis* with a declared activity of 2.4 AU/g and a density of 1.17 g/ml that fulfills the specifications of purity for food-grade enzymes recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

The reagents used in the experiments were of analytical or food-grade quality.

Analytical Methods

Fish waste samples were analyzed for moisture, crude protein, collagen, ether extract, crude fiber and ash. Moisture was determined by drying the sample to a constant weight in an oven at 105 °C [26]. Crude protein was determined by the analysis of the nitrogen content according to the semi-micro Kjeldhal technique (Kjeltec-System Foss Tecator) [27]; the protein content was calculated by multiplying the N content by a factor of 6.25 [28]. Total collagen content was measured by hydroxyproline determination according to the method of Bonnet and Kopp [29]. Fat in fish waste was determined as lipid content by petroleum ether extraction, HCl–hydrolysis and re-extraction (Soxtec_ HT-6 Foss Tecator) [30]. Ash was determined by pre-combustion on a hot plate, followed by calcination at 550 °C to a constant weight [26]. The crude fiber was determined by H_2SO_4 and KOH—hydrolysis followed by calcination at 475 °C to a constant weight (Fibertec_ M6 Foss Tecator), according to the European Community legislation, Commission Regulation (EC) No 152/2009 [31]. Carbohydrate was calculated by difference: it was determined by subtracting the sum percentage of moisture, protein, fat, ash and crude fiber [32]. The number of analyses for each determination was chosen to obtain the highest accuracy of the methods. The results were expressed as mean ± standard deviation.

Enzymatic Hydrolysis

Hydrolysis were carried out in 0.5 l cylindrical jacketed glass reaction vessels with magnetic stirring, temperature control and pH control, as shown in Fig. 1. A measured amount of fish waste was added to the reaction vessels containing distilled water to make a substrate concentration ranging from 5 to 45 g/l. Before hydrolysis, the solution was preheated to the reaction temperature and adjusted the pH using 2 N NaOH. The reaction was initiated by adding the enzyme (0.94–4.68 AU/1). During the process, the temperature was controlled using a thermostatic bath and pH was maintained by the addition of 2 N NaOH.

The controlled hydrolysis conditions were: pH = 8, temperature = 50 °C, time = 180 min and stirring speed = 300 rpm. Optimal pH and temperature values, corresponding to the maximum enzymatic activity and stability of Alcalase, were recommended by the enzyme supplier. The hydrolysis time was fixed at 180 min because longer times do not cause a significant increase in the degree of hydrolysis. A stirring speed of 300 rpm was chosen in order to keep homogeneous the reaction mixture and avoid vortices formation in the wells.

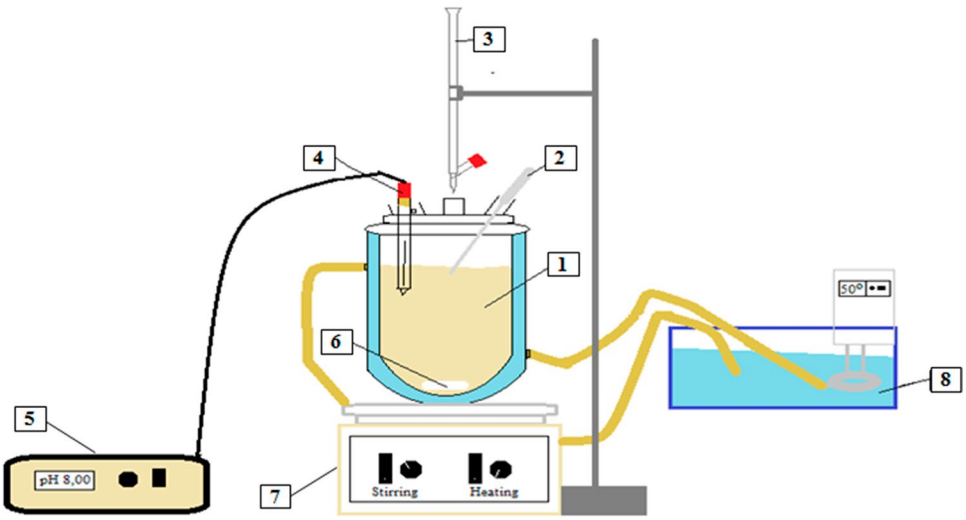

Fig. 1 Experimental set-up: (1) jacketed reactor, (2) thermometer, (3) burette, (4) pH electrode, (5) pH-meter, (6) stirrer bar, (7) magnetic stirrer and (8) thermostatic bath

Hydrolysis was monitored by measuring the extent of proteolytic degradation using the degree of hydrolysis (DH) according to the pH–stat method [28]:

$$DH\,(\%) = \frac{Number\ of\ peptide\ bonds\ cleaved}{Total\ number\ of\ peptide\ bonds} \times 100 \quad (1)$$

DH can be determined by using the following equation:

$$DH\,(\%) = \frac{B \cdot N_b}{M_p \cdot \alpha \cdot h_{tot}} \times 100 \quad (2)$$

where B is the alkali consumption during the hydrolysis (ml or l), N is the normality of the alkali, M_p is the initial mass of protein in the reactor (g or kg), α is the average degree of dissociation of the α-NH_2 groups released during the hydrolysis and h_{tot} is the total number of peptide bonds in the protein substrate (8.6 meqv/g or eqv/kg for fish protein, [28]), α varies with pH and temperature: at pH 8.0 and 50 °C of temperature, $1/\alpha = 1.1$ [28].

After the enzymatic hydrolysis, samples were placed into boiling water at 95–97 °C for 20 min with the purpose of deactivating the enzyme and pasteurizing the mixture (78 °C and 25 min are sufficient for pasteurization of the mixture [33]). Then, samples were centrifuged at 6000 rpm for 20 min and separated in three phases: lower phase (solid residue) containing the collagen, intermediate phase (supernatant) containing the protein hydrolysate, and the upper phase containing the separated oil. The residue was estimated for collagen recovery, the supernatant was estimated for solubilized protein (hydrolyzed protein) and upper-layer was estimated for oil recovery.

Collagen in residue and protein in the supernatant were analyzed as indicated in the section of "*Analytical Methods*". Oil was determined by measuring its volume; by multiplying the obtained volume by the fish oil density (0.919 g cm^{-3}), the weight of recovered oil was calculated.

All the tests were duplicated, and student's t-test was performed (significant level $p \leq 0.05$).

Products recovery was calculated as the percentage of total content in fresh fish waste according to the following equations:

$$Collagen\ recovery\,(\%) = \left[\frac{Collagen\ in\ the\ residue\,(g)}{Initial\ collagen\ in\ reactor\,(g)}\right] \times 100 \quad (3)$$

$$Hydrolysed\ protein\ recovery\,(\%) = \left[\frac{Protein\ in\ the\ supernatant\,(g)}{Initial\ protein\ in\ reactor\,(g)}\right] \times 100 \quad (4)$$

$$Oil recovery\,(\%) = \left[\frac{Oil in upper phase after the centrifugation (g)}{Initial fat in the reactor (g)}\right] \times 100 \quad (5)$$

Experimental Strategy

The enzymatic hydrolysis of fish waste protein has not been used so far to produce hydrolysates, oil, and collagen simultaneously. However, hydrolysis of food proteins has already been addressed by different authors and hence the variables that influence the kinetics of the reaction are well known: temperature, pH, the initial enzyme concentration (*Eo*) and the initial substrate concentration (*So*) [28]. Accordingly, in this work, pH and temperature were fixed at 8 and 50 °C, respectively, corresponding to the maximum enzymatic activity and stability of Alcalase, and the influences of *Eo*, *So* and *Eo/So* ratio on the by-products recovery were studied.

Four sets of analyses were carried out aimed to evaluate the performance of Alcalase at different initial concentrations of enzyme *Eo* and protein *So* in the reactor. In addition, tests were conducted to demonstrate the enzyme–substrate ratio (*Eo/So*) influence on the by-products recovery efficiency and the relationship between the degree of hydrolysis and the by-products recovery.

Results

Characterization of Samples

The fish waste used in this study had high moisture content (71.3 ± 0.6% w/w), in accordance with the literature where it is proved that fishes and shellfishes show a great content of water [34]. On a dry matter basis, waste was rich in proteins and lipids. Fish waste consisted of heads, skins, bones, viscera, shells and small whole fishes causing it to have high protein content (44.7 ± 0.6% w/w); these results agree with other studies showing that the protein content in fish waste varies between 35.9% w/w [35] and 57.9% w/w [5]. The main fishes observed in the waste were blue fishes with high-fat content that justifies the high percentage of lipids in samples (35.1 ± 2.1% w/w); according to the literature reviewed, fat content for fish waste is comprised between 19.1% w/w [5] and 52.5% w/w [35] which agrees with the obtained result. Although protein and lipids were the major components, the ash content reported was also high (19.6 ± 0.6% w/w), agreeing with literature data [35]. In fact, it is known that fish is an important source of minerals: heads and bones are an excellent source of many essential minerals such as calcium, phosphorus, potassium, iodine, selenium, zinc, iron, etc.[36]. Nevertheless, the content of fiber (0.0% w/w) and carbohydrates (0.6 ± 0.0% w/w) were negligible as expected at such waste [34]. Results obtained for total collagen content reported 5.9 ± 0.1% (w/w); this is coherent with the composition of fish waste since fish muscles have low collagen content [37] but fish skins are rich in it [38].

Influence of Alcalase Concentration

The gradual increase of the initial concentration of Alcalase (*Eo* from 0.94 AU/1 to 4.68 AU/1)—at a constant initial substrate concentration (*So* = 25 g of protein per liter)—increased the degree of hydrolysis values, and the oil and hydrolyzed protein recovery but not the collagen recovery (Fig. 2).

It was noted that a one-unit increase in the activity of Alcalase implied an increase of six-units in the degree of hydrolysis (Fig. 2a). This increase in the degree of hydrolysis corresponds to an increase in the concentration of hydrolyzed protein (Fig. 2c) and a decrease in the amount of collagen in the residue (Fig. 2d). This result is consistent because the presence of enzyme favors the hydrolysis, which generates an increase in the amount of produced hydrolysate (a one-unit increase in the enzyme activity means an increase of 75% in the production of hydrolyzed). Similar results were reported for the enzymatic hydrolysis of proteins from yellowfin tuna wastes using Alcalase [39]. On the other hand, collagen is more difficult to be hydrolyzed making another part remain not hydrolyzed in the solid phase so that the decrease in collagen by hydrolysis in that same range of enzyme activity was only 37%.

Regarding the oil recovery, an increase in the solubility of the protein after hydrolysis favors the separation of oil whose amount in the upper phase increases by 30% in the range of enzyme concentration studied, reaching a value higher than 75% of the oil contained in the waste (Fig. 2b). Higher solubility of hydrolysates in comparison to the solubility of waste is due to the modification in the secondary structure of fish protein during the enzymatic hydrolysis. The reduction of the molecular size of resulting peptides and the increase of newly exposed ionizable amino and carboxyl groups increase the hydrophilicity of the hydrolysates [40].

Influence of Protein Concentration

For a constant 0.84 UA/l concentration of Alcalase, the influence of the initial concentration of substrate in the range of 5–45 g/l was studied (Fig. 3). It was found that the number of broken peptide bonds increased with the concentration of substrate (as deduced from the increase of the alkali consumption); however, due to the higher initial number of peptide bonds, the degree of hydrolysis decreased as the *So* increased (Fig. 3a). This is due to the enzyme concentration remained constant during the experiments. At a constant Alcalase concentration and a lower concentration of the substrate, the substrate concentration is the limiting factor and the enzyme reaction rate increases as the initial substrate concentration increases. However, at higher concentrations as used in this study, the substrate often acts as a dead-end inhibitor by binding to the incorrect sites [41]. The hydrolyzed protein recovery decreased gradually as the initial substrate concentration increased. Comparing the reaction with 5–45 g/L, the hydrolyzed protein recovery decreased 43% (Fig. 3c). However, there was a rise of 86.19% in the collagen amount that remained in the solid phase (Fig. 3d). Moreover, as consequence of the lower protein solubilization, the amount of released oil decreased by 49% (Fig. 3b).

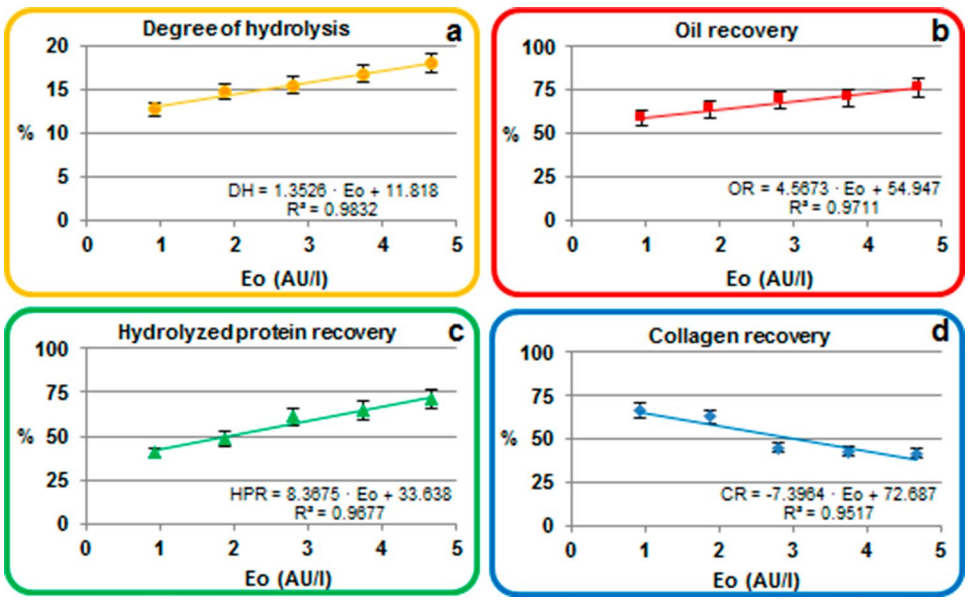

Fig. 2 Influence of Alcalase concentration on: **a** degree of hydrolysis [DH], **b** oil recovery [OR], **c** hydrolyzed protein recovery [HPR] and **d** collagen recovery [CR], at an initial substrate concentration of 25 g of protein per liter

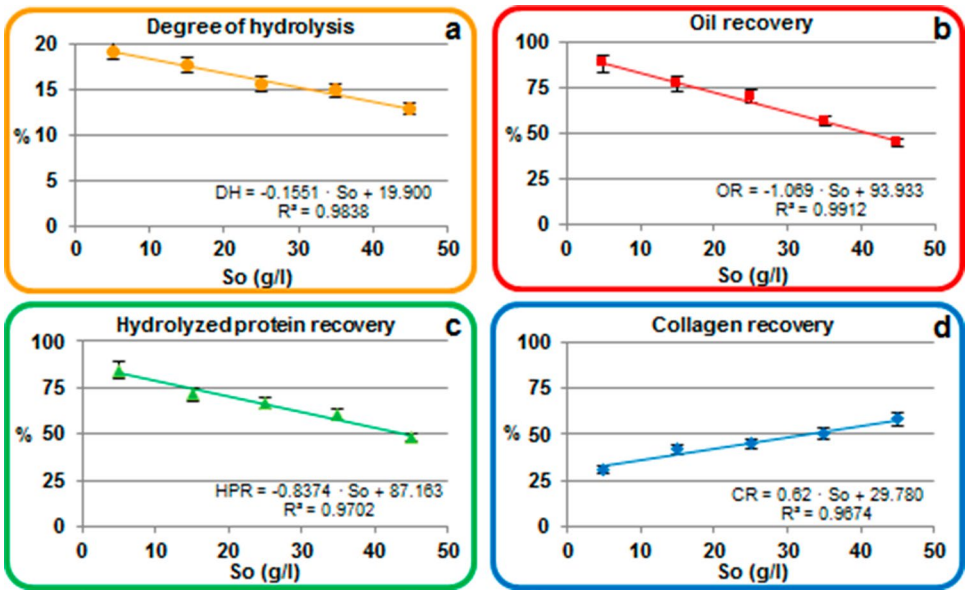

Fig. 3 Influence of substrate concentration on: **a** degree of hydrolysis [DH], **b** oil recovery [OR], **c** hydrolyzed protein recovery [HPR] and d collagen recovery [CR], at an initial Alcalase concentration of 0.84 UA/l

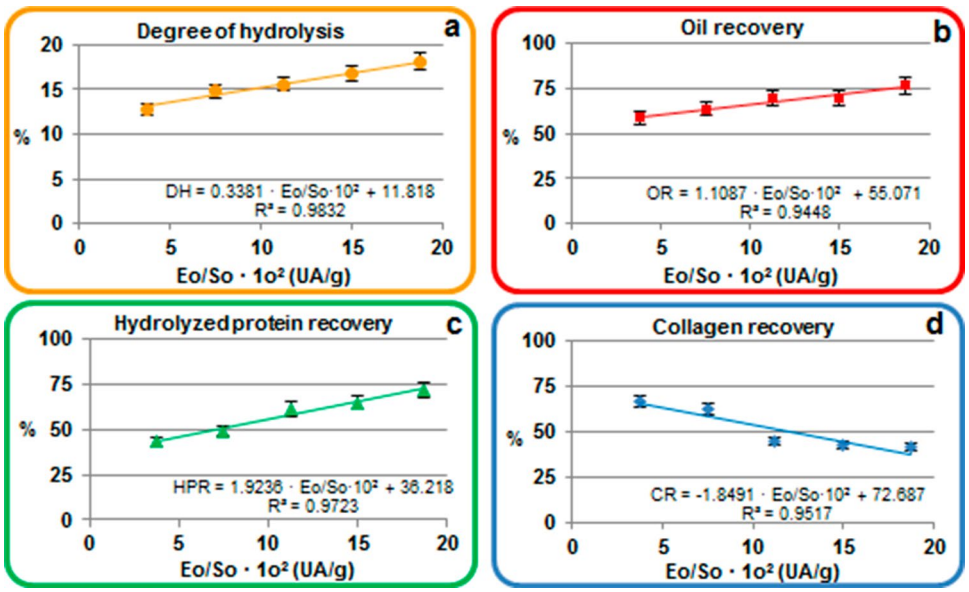

Fig. 4 Degree of hydrolysis and by-products recovery, at constant 0.19 UA/g enzyme/substrate ratio, for different enzyme and substrate concentrations

Influence of Enzyme/Substrate Ratio

The fact that the increase of the initial concentration of substrate, keeping enzyme concentration constant, did not favor the recovery of by-products seems to indicate that the enzyme/substrate ratio is a significant process variable. To verify this assertion, experiments with different initial concentrations of enzyme and substrate were carried out, keeping constant the *Eo/So* ratio. Results obtained at the same *Eo/So* ratio showed identical values as much for the degree of hydrolysis as for the protein hydrolysate, collagen and oil recoveries (Fig. 4). It proves that the ratio between the initial concentration of the enzyme and the initial concentration of the substrate is the most influential variable in the process for the studied operating conditions.

To check the influence of *Eo/So* ratio on by-products recovery, experiments were performed at different ratios, from 0.03 to 0.20 AU/g (Fig. 5). As was expected, based on previous experiments in sections entitled "*Influence of Alcalase concentration*" and "*Influence of protein concentration*", the amounts of the recovered by-products are closely related to the enzyme/substrate ratio. The increasing of the ratio (obtained either from a higher enzyme concentration or a lower substrate concentration) resulted in a hydrolysate with a higher degree of hydrolysis. For a rise of only 0.045 AU/g, the hydrolysis degree increased 41.4%

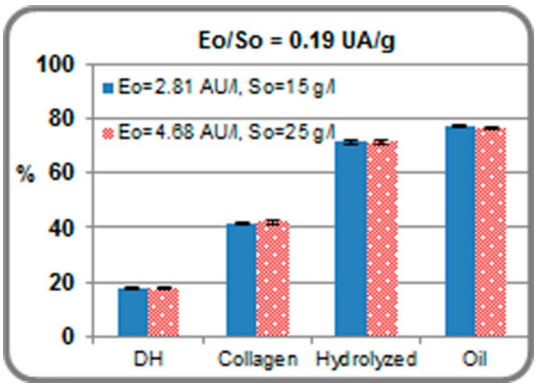

Fig. 5 Influence of different enzyme/substrate ratios on: **a** degree of hydrolysis [DH], **b** oil recovery [OR], **c** hydrolyzed protein recovery [HPR] and **d** collagen recovery [CR]

(Fig. 5a), the hydrolyzed protein increased 100% (Fig. 5c), the collagen recovery decreased 59.7% (Fig. 5d) and the oil recovery increased 56.2% (Fig. 5b). It is explained by Silva et al. [42], who reported that, at higher relative enzyme concentrations, there were more active sites available on the enzyme to hydrolyze the substrate, resulting in greater cleavage of the peptide bonds, and consequently higher dissolution of the proteins.

Relationship Between Degree of Hydrolysis and Recovery of By-Products

Linear regression analysis was applied to assess the relationships between the degree of hydrolysis and the recovery of oil, hydrolyzed proteins and collagen (Fig. 6). A direct linear relationship between the degree of hydrolysis and the formation of protein hydrolysates and oil recovery was observed. The determination coefficients near 1 for both hydrolysate (0.991) and oil (0.991) indicate that hydrolyzed protein and oil recovery had a general tendency to increase as the degree of hydrolysis increased. As it can be seen in Fig. 6a, b, the regression lines have an ordinate at the origin of 0. It is what it would be logically expected since it is evident that for DH = 0 the production of hydrolysate and the recovery of oil are not possible. This fact indicates that the obtained relationships are an accurate adjustment set for calculating the causal effect of DH on hydrolysate and oil production.

However, an inverse linear relationship can be observed between the degree of hydrolysis and the collagen content in the residue. In this case, the determination coefficient near 1 implies that collagen recovery had a clear tendency to decrease as the degree of hydrolysis decreased. As expected, now the regression line has an ordinate at the origin of 100, meaning that for DH = 0 all collagen remains in the solid part since it is not hydrolyzed. This result confirms the reliability of the relationship obtained to determine the amount of unhydrolyzed collagen from the degree of hydrolysis.

According to the linear regression equations (Fig. 6), the optimal yield for the by-products recovery could be achieved with a degree of hydrolysis of 25% for which 430 g of hydrolyzed protein, 10 g of recovered collagen and 350 g of fish oil could be obtained per kg of fish waste. Considering the average market prices of protein hydrolysates, collagen and fish oil, and the main operating costs (corresponding to energy and enzyme costs), a sale revenue of 10.6 €/kg of fish waste could be generated. Table 1 shows the detailed data for calculating the sale revenue. Moreover, the use of fish waste as raw material for by-product recovery resulted in a 79% reduction in waste disposed to landfills.

For this degree of hydrolysis, the average peptide chain length, PCL, estimated as PCL = 100/DH [28], was four corresponding to peptides of about 500 Da of average molecular

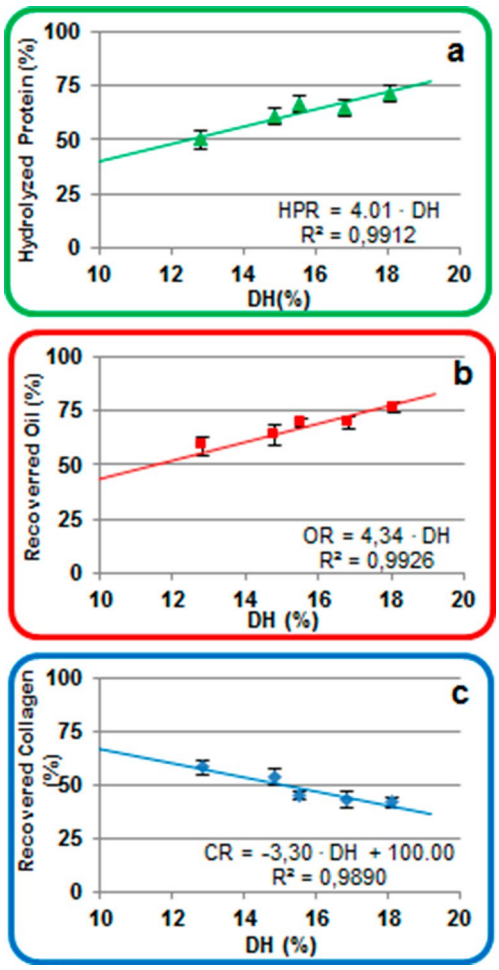

Fig. 6 Correlation between the degree of hydrolysis [DH] and: **a** hydrolyzed protein recovery [HPR], **b** oil recovery [OR] and **c** collagen recovery [CR]

Table 1 Income and cost of production

Income[a]	
Protein hydrolysate	
$kg/kg_{fish\ waste}$	0.43
€/kg	22.11
$€/kg_{fish\ waste}$	9.51
Collagen	
$kg/kg_{fish\ waste}$	0.01
€/kg	16.01
$€/kg_{fish\ waste}$	0.16
Oil	
$kg/kg_{fish\ waste}$	0.35
€/kg	4.99
$€/kg_{fish\ waste}$	1.74
Total ($€/kg_{fish\ waste}$)	11.41
Cost[b]	
Enzyme	
$AU/kg_{fish\ waste}$	49.14
€/AU	$1.53 \cdot 10^{-2}$
$€/kg_{fish\ waste}$	0.75
Energy	
$kJ/kg_{fish\ waste}$	1454.01
€/kJ	$2.88 \cdot 10^{-5}$
$€/kg_{fish\ waste}$	0.04
Total ($€/kg_{fish\ waste}$)	0.79
Sale revenue	
$€ /kg_{fish\ waste}$	10.62

NaOH cost is negligible compared to enzyme and energy cost

[a]Average market prices

[b]According to updated data of Esteban [43]

weight. From a nutritional perspective, these peptides are more bio-available than proteins or free amino acids, less allergenic than peptides with an average molecular weight higher than 2000 Da [44] and there is a lack of the bitter taste that peptides with an average molecular weight higher than 1000 Da [45].

Conclusions

The hydrolysis of fish waste with Alcalase 2.4 L resulted in the production of protein hydrolysates and the recovery of collagen and oil. The protein hydrolysates obtained were rich in low molecular weight peptides suitable for use as nutraceutical ingredients. Collagen and fish oil recovered were also high value-added products that could be widely marketed. All of them have potential applications in the pharmaceutical and cosmetic industries.

It was proved that the enzyme/substrate ratio was the main factor controlling the hydrolysis rate at the assayed conditions. Linear relationships between the degree of hydrolysis and the obtained products were achieved. These relationships allow the prediction of the amounts of by-products that can be obtained from fish waste at different operating conditions by only knowing the degree of hydrolysis reached.

Thus, enzymatic hydrolysis is a promising technology with great potential to increase the fish processing plant revenue, by obtaining simultaneously protein hydrolysates, collagen and oil from fish waste, and to reduce the amount of organic matter disposed of in landfills, making these industries more sustainable.

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