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ENZYMATIC HYDROLYSIS AS A MEANS OF RECOVERING BOVINE HIDES: LABORATORY AND MEDIUM SCALE TRIALS, CHARACTERIZATION OF HYDROLYSATES AND SCALE-UP TO SEMI-INDUSTRIAL SCALE

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ABSTRACT

A current hot topic in the field of environmental protection and development of valuable new products is represented by the recovery of food waste from the meat sector. The present study focused on the recovery of bovine hides by means of a sustainable method. Enzymatic hydrolysis was selected as a potential green methodology for use in the production of protein hydrolysates to be applied on an industrial scale. For this purpose, the enzymatic hydrolysis of bovine hides with Alcalase was investigated following a multiscale approach: lab-scale, medium-scale and semi-industrial pilot plant. Alcalase proved to be highly efficient due to its ability to degrade collagen, the main protein of hides. Under optimized conditions, the hydrolysis of hides at lab- and medium-scales resulted in approx. 85% protein solubilisation after 6 hours, with a consistent release of free amino acids and a degree of hydrolysis of 17-19%. However, in the pilot plant, solubilisation decreased due to difficulties in mixing bovine hides in the reactor, which was compensated with a longer reaction time to achieve virtually total protein solubilisation (~98%). The present data therefore demonstrate the applicability of the process at semi-industrial scale in protein recovery with a reduced amount of waste by-products.

1. INTRODUCTION

Beef is a primary source of energy and proteins. However, only 44% of live animal weight ends up in the food chain (Russ and Meyer-Pittroff, 2004), while the remaining 56% is usually discharged. This waste is composed of edible (e.g. offal) and non-edible parts (e.g. specified risk materials). Due to the high content of organic material, the utilisation and disposal of this type of waste is difficult to manage. However, the identification of suitable strategies aimed at valorising meat by-products is of particular interest from an economic and environmental point of view. Indeed, these by-products could be exploited as a cheap source of valuable compounds, and subsequently recovered and recycled within the food chain as functional additives or ingredients in a series of food or feed products (Lynch et al., 2018). Numerous studies have focused on the recycling and conversion of meat processing waste through sustainable-environmentally-friendly methodologies (Jayathilakan et al., 2010). Nowadays, there is a growing interest in studying these non-meat products with a view to recovering additional value from the meat processing chain by means of a cleaner methodology.

The main focus of the present study was a specific by-product of the meat processing procedure, i.e. bovine hides. Bovine hides are often used as a raw material in leather processing. However, the low added value fails to make this solution particularly appealing for meat producers. The percentage of bovine hides produced during slaughtering is ~ 7% of the animal weight (Mullen et al., 2010); only ~ 25% of this part is converted into leather (Cooper et al., 2011), with the remaining part considered a waste or low-quality by-product (Notarnicola et al., 2011). This waste (i.e. trimmings, fleshings and damaged hides), unsuitable for the leather industry, is discarded despite its potential value, and is forwarded to landfill, thereby resulting in a significant emission of methane and additional costs for the producers (Notarnicola et al., 2011). To avert

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this tremendous impact on the environment, innovative strategies and clean methodologies to be used in recovering these by-products should be identified.

Bovine hides are rich in proteins and could potentially be used as a feedstock in the production of protein supplements destined to a range of end-uses (Mullen et al., 2010). Due to their high collagen content, bovine hides are generally converted into gelatines by thermal denaturation and/or chemical degradation (Bajza and Vrcek, 2000). Gelatine production is conventionally based on chemical degradation and solubilisation of collagen (acidic or alkaline). In addition to a high environmental burden, this traditional method of collagen extraction is extremely lengthy and requires the use of strong polluting chemicals, resulting in the chemical degradation of proteins and amino acid modification (Damrongsakkul et al., 2008; Morimura et al., 2002; Ravindran and Bryden, 2005). Conversely, the advantages of enzymatic hydrolysis include: shorter timing, more benign conditions, high yields, process safety, low refining processes and few or no undesirable side reactions (amino acid preservation) (Raveendran et al., 2018; Alvarez et al., 2012). Moreover, the physicochemical, organoleptic and functional properties of the initial protein substrates can be improved by means of enzymatic hydrolysis (Awuor et al., 2017). In particular, the utilization of specific proteases affects the nutritional, bioactive and functional properties of food proteins: improving digestibility, sensory properties, antioxidant capability or reducing allergenic compounds (Tavano, 2013). Furthermore, enzyme specificity can be channelled towards the production of protein hydrolysates with better-defined chemical and nutritional characteristics (Castro et al, 2011).

For these reasons, enzymes are widely used in various industrial sectors such as food, detergent, paper and textile industries.

Based on the above premises, this paper proposes, for the first time, enzymatic hydrolysis as a sustainable alternative methodology for use in the recovery of protein hydrolysates from bovine hides. Due to the high demand for readily-available amino acids in soil fertilizers, in food/feed preparation and in the pharmaceutical industry, particular attention was focused on the production of protein hydrolysates with a high degree of hydrolysis, with the aim of obtaining a high amount of free amino acids and small peptides. Accordingly, Alcalase was selected as the hydrolysing enzyme, particularly in view of its ability to degrade collagen (the main component of hides) (Anzani et al, 2017a,b). The process kinetics were evaluated at lab- and medium-scales, and the hydrolysates obtained were fully characterized. This method was subsequently further optimized for the first time, in a semi-industrial pilot plant, verifying its applicability at industrial-scale to produce protein hydrolysates for use in high quality applications.

2. MATERIALS AND METHODS

Samples, constituted by pieces of bovine hides, were provided by Inalca Industria Alimentare Carni SpA (Castelvetro di Modena, Italy).

2.1 Reagents and solvents

Alcalase from Bacillus licheniformis (2.59 U/g, SL-BL2953V), sodium dihydrogen phosphate, hydrochloric acid, acetonitrile, dichloromethane, N-acetyl-L-cysteine, sodium tetraborate decahydrate, sodium thiosulfate, DL-isoleucine, DL-norleucine, trifluoroacetic anhydride, sulfuric acid, formic acid, acetic acid and trifluoroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Copper oxide was obtained from Carlo Erba (Milan, Italy).

O-phthalaldehyde and boric acid were bought from Fluka (Buchs, Switzerland).

Kjeldahl tablets defoamers and catalyst 3.5 g/tablet was purchased from Merck (Darmstadt, Germany). AccQ-Fluor reagent kit was obtained from Waters (Milford, Ma, U.S.A).

Soy lecithin was purchased from a local market.

2.2 Enzymatic hydrolysis protocol at lab- and medium-scale

The processes were performed in a 5 L reactor equipped with a heating shell and agitator blade. The experiments were performed at lab- and medium-scales; the details are reported in Table 1.

The protocol employed has been described in a previous publication (Anzani et al., 2017a), with several modifications. The two washing and degreasing steps were applied to the pieces of hide. In the first step 2% Na_2CO_3 and 1% of soy lecithin were added, and in the second 1% Na_2CO_3 and 1.5% of soy lecithin. For both steps the temperature was set at 60°C for 30 min of agitation at a rotation speed of 100 rpm. At the end of both washings, the wastewaters were discarded. The hides were subsequently swollen to remove the traces of alkali with an ulterior wash. This stage required 1 hour of agitation at 60° in a solution of Na_2HPO_4 10 mmol L^{-1}. After the swollen step, the solution was discarded.

The hide suspension was then prepared in a buffer solution (Na_2HPO_4 10 mmol L^{-1}) and the pH of the solution monitored. The enzymatic hydrolysis reaction commenced at pH 7-7.5 with addition of the enzyme as 11.02 U/100 g of

TABLE 1: Amount of bovine hides, solution used in the enzymatic protocols and hydrolysates volume recovered.

Experiment	Bovine hides (g)	Volume of added buffer (mL)	Volume of final hydrolysate after reaction (mL)
Lab scale hydromodule 1:2	500	1000	1460
Medium scale hydromodule 1:2	1500	3000	4400
Lab scale hydromodule 1:3	422	1265	1670
Medium scale hydromodule 1:3	1000	3000	3950

proteins at 60°C under stirring. The enzyme used (Alcalase from *Bacillus licheniformis*) was selected based on previous studies (Anzani et al, 2017a; Anzani et al, 2017b). The digestion was sampled every 30 min for 6 hours and then boiled (10 minutes, 90°) to inactivate the enzyme. The final volume was measured with graduate cylinders.

2.3 Determination of protein content in broths and hide samples

In line with standard procedures A. O. A. C. (2002), protein content was evaluated by means of a rapid Kjeldahl system (VELP SCIENTIFICA DKL heating digester and VELP SCIENTIFICA UDK 139 semiautomatic distillation unit (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy)). The protein amount was determined from total nitrogen content (%N x 6.25). In particular, in evaluation of the protein content of the raw material, 12 pieces were analysed in order to take into account the biological variability of hides. In the case of broths, protein content was estimated after centrifugation at 4000 rpm for 30 min on the supernatant. The percentage of solubilised proteins was determined as grams of proteins present in the supernatant over the total protein content (grams) in the starting material (calculated from the average amount of the protein of bovine hides).

2.4 Determination of the degree of hydrolysis (DH) using OPA (o-phthaldialdehyde) method

The degree of hydrolysis was determined and calculated following the protocol already proposed in our previous research paper (Anzani et al, 2017b).

2.5 Quantification of free and total amino acids by means of high-performance liquid chromatography with fluorescence detection (HPLC/FLD) after AccQ•Tag derivatisation

The determination of free amino acids was executed following our previous protocol (Anzani et al., 2017b). In brief, 100 µL of samples were employed. Samples were mixed with 34 µL of norleucine (5 mM in deionized water), which was added as internal standard and the volume made up to 1 mL with deionized water. In order to perform a calibration curve, a standard solution was prepared: 40 µL of norleucine (2.5 mM in HCl 0.1 N), 40 µL of amino acids hydrolysate standard mixture (Sigma Aldrich, Saint Luis, Missouri, U.S.A), 40 µL of cysteic acid (2.5 mM in HCl 0.1 N), 40 µl of hydroxyproline (2.5 mM in HCl 0.1 N) and 840 µL of deionized water were mixed. Then, 10 µL of either samples or standard solution were transferred into 1.5 mL tubes and 70 µL of borate buffer (obtained from AccQ•Tag Ultra Derivatisation Kit (Waters, Milford, Massachusetts, U.S.A)) and the solution was vortexed. 20 µL of reconstituted AccQ•Tag reagent (Waters, Milford, Massachusetts, U.S.A) were finally added and the mixture was vortexed again for few seconds. The tube was left closed at room temperature for 1 minute and was then warmed up in a heated bath at 55°C for 10 min. The derivatised samples were diluted with 400 µL of deionised water before injection into the HPLC system.

Samples were analysed using an Alliance 2695 separation system with AccQ•Tag amino acid analysis column (3.9mmX150mm), (Waters, Milford, Massachusetts, U.S.A). The column was thermostated at 37°C and the flow rate set at 1.0 mL/min. The injection volume of samples was 10 µL, while standard calibration solution was injected at several volumes: 2.5, 5, 10, 15, 20, 25 and 30 µL, corresponding to 5, 10, 20, 30, 40, 50 and 60 pmoles of amino acids hydrolysate standard mixture (Sigma Aldrich, Saint Luis, Missouri, U.S.A) injected. Mobile phase A consisted of AccQ•Tag eluent A (100 mL AccQ•Tag A concentrate+1L deionised water). Mobile phase B was a 60:40 (V/V) solution of acetonitrile and deionized water, respectively. Gradient elution was performed according to the following steps: 0 min 100% A, 1 min 97% A, 13 min 93% A, 18 min 90% A, 38 min 67% A, 51 min 67% A, plus washing step and reconditioning. Detection was carried out using a Waters 470 fluorescence detector (λ excitation = 250 nm and λ emission = 395 nm).

Quantitative analysis was carried out using the internal standard method. The analysis was performed in triplicate.

2.6 Peptide identification by LTQ-Orbitrap analyses

For high-resolution mass spectrometry, a µHPLC DIONEX Ultimate3000 interfaced with an LTQ-Orbitrap XL Thermo Fisher Scientific was used (Thermo Fisher Scientific, Waltham, MA, U.S.A). (Formic Acid); eluent B: ACN (Acetonitrile) + 0.1% FA; flow: 5 µL/min, gradient: 0-4 min from 100% A to 95% A, 4-60 min from 95% A to 50% A, 60-62 min from 50% A to 10% A, 62-72 min 10% A, 72-74 min from 10% A to 95% A, 74-90 min 95% A; analysis time (min): 90; column temperature (°C): 30; injection volume (µL): 5; acquisition time (min): 0-75; ionization mode: ESI+; scan range (m/z): 200-1,800; source voltage (kV): 3.5; capillary voltage (kV): 35; source temperature (°C): 275. Scan event details: (Fourier transform) FTMS+p res=30,000 or (250.0-2000.0); (ion trap) ITMS+cDep MS/MS Most intense ion form; activation type: CID; isolation width: 2.00; normalized coll. energy: 35.0; default charge state: 2; activation Q: 0.250; activation time: 30.000; dynamic exclusion enabled; repeat count: 2; repeat duration (s): 10.00; exclusion duration (s):30.00. Charge state rejection: enabled; unassigned charge states: rejected; charge state 1: rejected; charge state 2: not rejected; charge state 3: not rejected; charge states 4+: not rejected; ion signal threshold: 10,000. Proteome DiscovererTM software (Thermo Fisher Scientific) was used for the identification of peptides.

2.7 Protocol scale-up in a semi-industrial plant

The scale-up reaction was performed at Po.Te.Co s.c.r.l Technogical center (Via San Tommaso, 119/121/123 56029 - Santa Croce sull´Arno (Pisa), Italy). The equipment used for these experiments had already been employed in our previous study (Anzani et al., 2017a). In particular, a drum (80 cm diameter, 55 cm length, capacity 70 L) was equipped with a cooling/heating plant and fitted with a system for continuous temperature measurement (Italprogetti srl, Sospiro, Cremona, Italy). 10 kg of pieces of hides were employed and a 30 L volume of solution used. The protocol used is described in section 2.2. In the pilot plant scale-up the rotation speed was 10 rpm, the samples were collected

every 30 min for 5 hours, and the enzyme was inactivated at 90° for 10 minutes. Following the result of this semi-industrial experiment, a further trial was carried out under the same experimental conditions (10 kg of pieces of hides, 30 L of solution) using a longer hydrolysis time (16 hours). The final volumes of hydrolysates in the large-scale reactor were calculated by immersion of a graduated dipstick into the reactor and using the height of the liquid to calculate the total volume.

3. RESULTS AND DISCUSSION

The protein content in the bovine hides, used as starting material, was determined by complete digestion and Kjeldahl analysis. The protein amount was 29.4 ± 3.6 g/100g hides (on wet basis), in agreement with Arunachalam and Saritha (2009). This value was considered as a reference for the calculations of the percentage of the protein solubilised during enzymatic hydrolysis and for the amount of enzyme added as related to the protein amount.

3.1 Enzymatic hydrolysis at lab- and medium-scale

Alcalase was chosen as the enzyme to be utilised in the hydrolysis process due to its ability to degrade collagen (Anzani et al, 2017a; Fu et al, 2017), its low cost and food grade (Doucet et al., 2003; Kristinsson and Rasco, 2000; Muzaifa et al., 2012; Saidi et al., 2016). Alcalase has been used previously to produce soluble hydrolysates from different raw materials (Anzani et al., 2017b; Sbroggio et al., 2016; Wisuthiphaet et al., 2016; Haslaniza et al., 2013). In fact, 60% of enzymes used for industrial purposes derive from proteases such as Alcalase (Singh et al., 2016). Due to the presence of a series of proteases with different specificities, this broad specificity enzyme, capable of hydrolysing the majority of peptide bonds, was used (Doucet et al., 2003). Indeed, compared to other enzymes (e.g. pepsin and trypsin), Alcalase usually generates protein hydrolysates with a high DH.

For the experiments, the enzyme was used at 11.02 U/100g of proteins and, in line with manufacturer indications of optimum temperature, the process temperature was selected at 60°C in order to obtain the best hydrolysis (Sigma-Aldrich). Furthermore, the results obtained in a previous study carried out by our research group revealed how Alcalase at 40°C resulted solely in dehairing of the hides, but not in their complete dissolution (Anzani et al., 2017a). Again in line with manufacturer (Sigma-Aldrich) indications (which showed for Alcalase a broad optimum between 6.5 and 8.5) and our own previously obtained results (Anzani et al., 2017a), the working pH was set at 7.

The hydrolysis reaction was only optimized in respect of time (evaluated during the process by continuous sampling of hydrolysates), and of two different hide/buffer solution ratios (defined as hydromodule), considering 1:2 and 1:3 ratios. Although the 1:1 ratio was preferable from an industrial point of view (low amount of water required and potentially evaporated at the end of the reaction), it was not considered feasible due to mixing problems previously observed in the 1:2 trial. Indeed, the high number of hides compared to the amount of buffer solution may result in increased friction in blade rotation, and potential block of the reactor.

The kinetic profiles of enzymatic hydrolysis were monitored in both cases (1:2 and 1:3 hydromodule) by characterising the soluble protein content released in solution during the reaction. Figure 1 shows the kinetic profile ob-

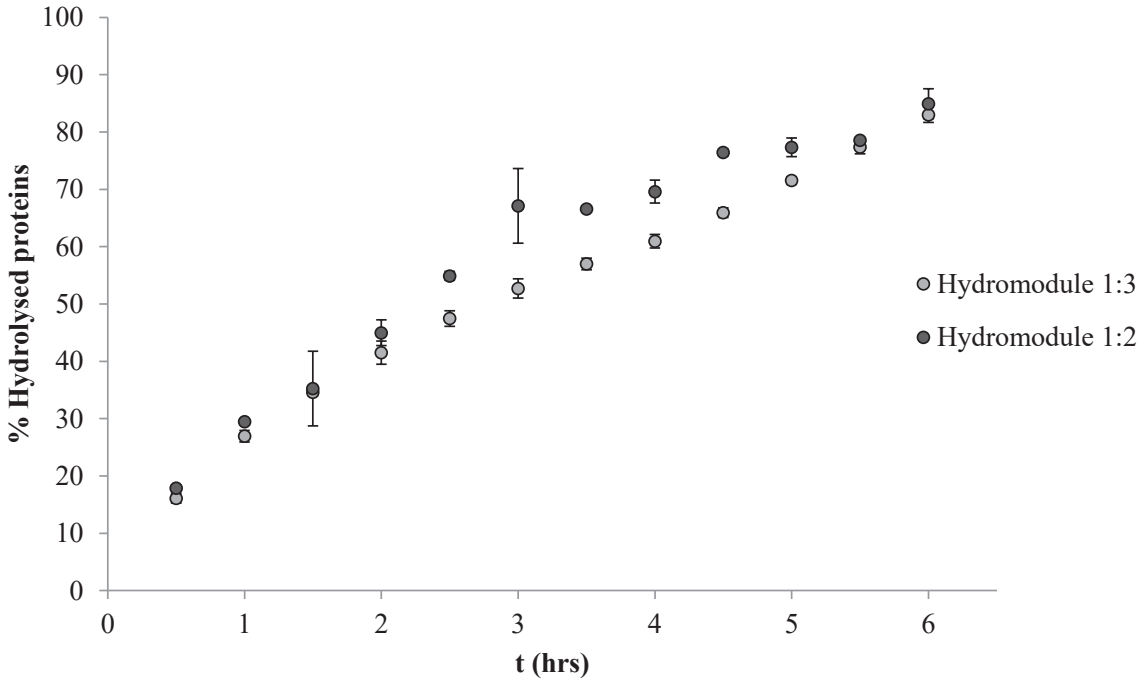

FIGURE 1: Kinetic profile of the percentage of solubilised proteins at different substrate/buffer solution ratios, over the total amount of proteins, as function of time.

tained by measuring protein concentration in solution at different intervals (determined by Kjeldahl analysis of the solubilized nitrogen). The kinetic profile of both hides/water ratios showed similar trends in both cases, with a sharp increase of the solubilised nitrogen starting to level out after 5-6 hours.

At the end of the reaction (after 6 hours), solubilized proteins (evaluated by Kjeldahl analysis of hydrolysates) with 1:2 hydromodule resulted in a concentration of 87 ± 1 mg/ml at small scale and 84 ± 3 mg/ml at medium scale. Considering the volume increase at the end of the reaction (see Table 1), this value corresponded to 127 ± 2 g and 370 ± 10 g of solubilised proteins respectively, and ~85% of protein solubilisation in both cases. In the experiment with a 1:3 hydromodule, at the end of the reaction, solubilised proteins were 60 ± 1 mg/ml at small-scale and 61 ± 1 mg/ml at medium-scale. Considering the volume increase at the end of the reaction (see Table 1), these values corresponded to 100 ± 2 g and 240 ± 4 g of solubilised proteins respectively, and to 82% of protein solubilisation in both cases. An important parameter in the proteolytic reaction is the degree of hydrolysis, which is closely related to the functional properties of the final hydrolysates (Wouters et al., 2016; Segura-Campos et al, 2010) and should be closely monitored. Moreover, the degree of hydrolysis is based on the number of peptide bonds cleaved into the free amino acids present in solution (Haslaniza et al., 2013). In particular, over time the DH increases due to the augmented release of free N-species in the solution (Figure 2), in agreement with previous studies (Dong et al., 2008; Merz et al., 2016; Sbroggio et al., 2016).

OPA analysis of the solutions indicated an extensive protein hydrolysis, reaching a degree of hydrolysis of ~20% (calculated on solubilized proteins) in both water/substrate ratios (Figure 2), although with differences between the two conditions. This is in agreement with previous studies showing that the %DH with Alcalase ranged between 16 and 25% (Merz et al, 2016; Guerard et al, 2001; Demirhan et al, 2011, Anzani et al, 2017b). In particular, in the hydromodule 1:2, the higher substrate concentration lead to a higher release of N-species during the first hour, compared to the other ratio, but with a lower value at the end of the reaction, (%DH=17.3 ± 0.1). Conversely, the more diluted solution with hydromodule 1:3, generated more N-free species at the end of the hydrolysis reaction (%DH = 19.2 ± 0.2), consistent with the findings of Butrè et al. (2014a). The latter authors demonstrated that water availability plays a crucial role in the hydrolysis of proteins (at constant enzyme/substrate ratio) by increasing protein concentration and decreasing the overall rate of enzymatic protein hydrolysis. Due to the high DH (>10%), these hydrolysates can be used in nutritional supplements or medical diets (Segura-Campos et al.,2010).

Since Alcalase is known to generate many free amino acids, as confirmed by the high DH observed, free amino acid content was evaluated by chromatographic methods, as shown in Figure 3 (details in the experimental section).

Analysis of free amino acids indicated a continuous increase of this species over time, together with solubilized nitrogen, reaching final amounts at the end of the reaction of 10.3 mg/ml for hydromodule 1:2 and 7.7 mg/ml for hydromodule 1:3. It is interesting to notice that the experiments producing the highest degree of hydrolysis lead to the lowest amount of free amino acids, and vice versa. This indicates that, on average, the 1:2 ratio leads to a higher amount of free amino acids, but also to longer peptides, whereas the experiment performed with hydromodule 1:3 results in fewer free amino acids but shorter peptides. Indeed, differences in the amount of free amino acids may derive from the influence of the hydromodule (or substrate

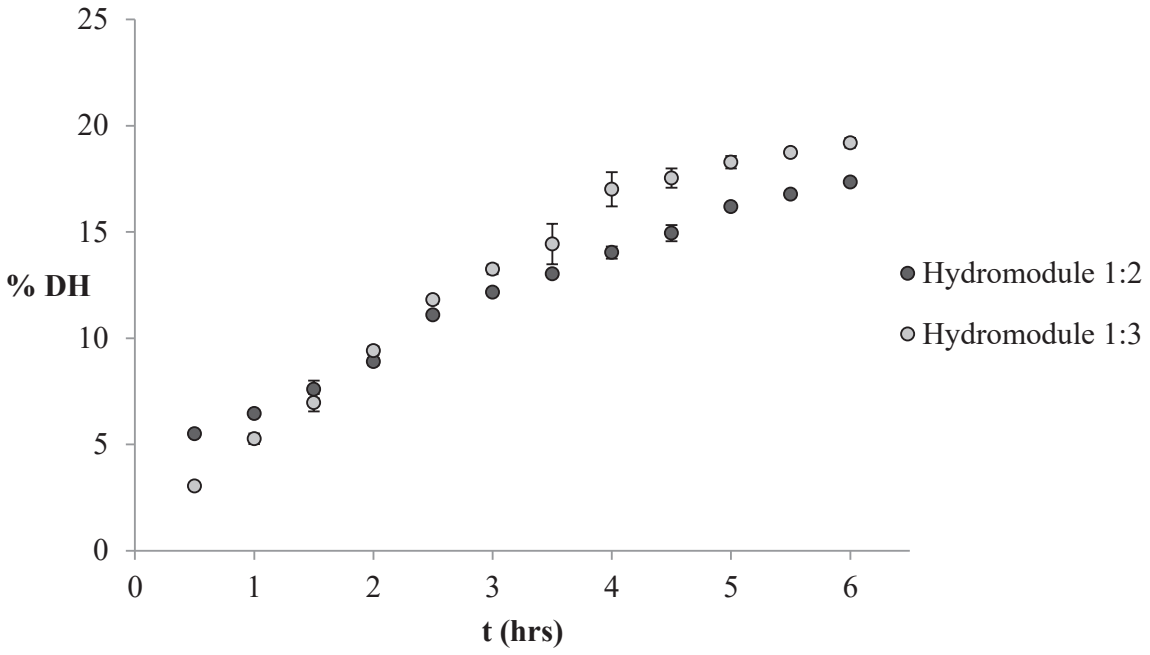

FIGURE 2: Kinetic profile of the percentage of the degree of hydrolysis at different substrate: buffer solution ratios, as function of time.

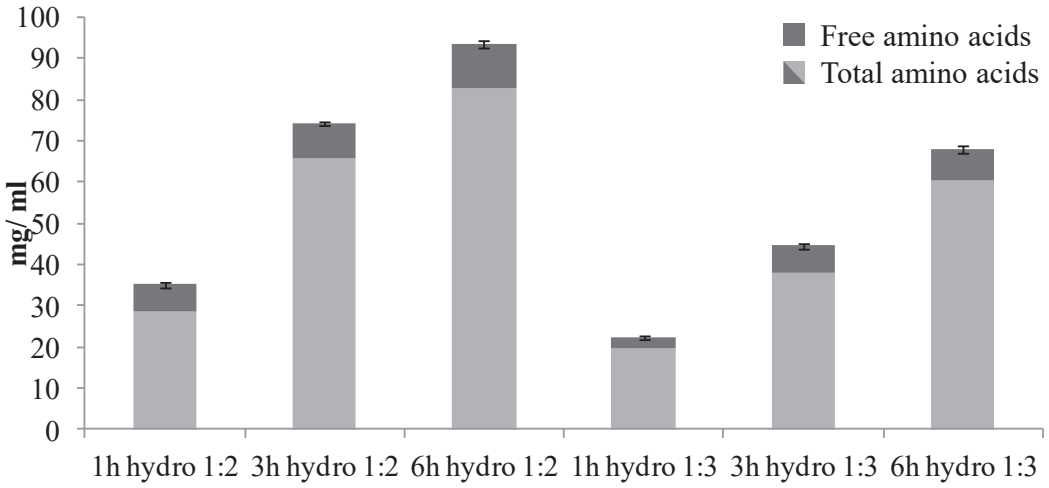

FIGURE 3: Distribution of free amino acids as compared to total nitrogen in the broths after enzymatic hydrolysis. Sum of the total nitrogen, determined by Kjeldahl method (full bar), and free amino acids determined by LC (darker part of the bars). By difference, the lower part of the bar, in a lighter grey, indicates the nitrogen fraction which is not part of the free amino acidic pool, thus amino acids contained in soluble peptides and proteins.

concentration) on both the hydrolysis mechanism and on DH (Butrè et al., 2014b). As a consequence, the hydromodule is an important parameter and should be taken into consideration in tailoring composition of hydrolysates.

To further understand the mechanism underlying the proteolytic process, proteomic analyses were performed on the solubilized nitrogen fraction. LTQ-Orbitrap analyses of the peptides present in the hydrolysates are illustrated in Table 2.

The data obtained revealed how peptides were derived mainly from collagen in both experiments. This indicated that the high efficiency of Alcalase in solubilizing hides is due largely to its ability to degrade collagen, as observed previously (Anzani et al., 2017b).

3.2 Enzymatic hydrolysis at semi-industrial scale

A scaled-up experiment was performed to investigate the applicability of this process in a semi-industrial plant, (10 kg of hide pieces in a 30 L solution). The semi-industrial plant employed was constituted by an agitation drum with an integrated heating/cooling system. The rotation speed was set at 10 rpm, due to equipment limitations. This implied that the mixing efficiency was expected to be much less than that obtained at lab- and medium-scale. The enzyme of choice (Alcalase), the quantity used (0.0324 U/g of bovine hides), optimal temperature (60°) and all other details of the previously applied protocol remained the same. Although both hydromodules yielded good results in terms of protein solubilisation, hydromodule 1:3 was selected in view of the difficulty experienced by the stirring blade in the experiments performed with hydromodule 1:2, where the rotation of the blade was hampered by the large amount of hides. Given the reduced mixing efficiency expected at industrial scale, a more diluted solution was thought to be needed to achieve the same efficiency in protein solubilisation. The experiment protocol was designed to be accommodated within a full working day (9 hours). In view of the time lag needed (to warm up the reactor (30 L at 60°C), and for pH adjustment), the allowed hydrolysis reaction time was 5 hours to be fully comparable to that used in lab- and medium-scale experiments.

In this first experiment, hide solubilisation efficiency

TABLE 2: Identification by LTQ-Orbitrap of the main peptides present in the solutions derived from enzymatic cleavage on bovine hides, and of the proteins of origin. Proteins are listed in order according to the highest number of peptides generated.

Sample	Protein	Score	N° peptides
Hydromodule 1:3	Collagen alpha-1(I) chain	914	231
	Collagen, type III, alpha 1	477	130
	Collagen alpha-2(I) chain	398	119
	Elastin	101	53
Hydromodule 1:2	Collagen alpha-1(I) chain	967	250
	Collagen, type III, alpha 1	513	129
	Collagen alpha-2(I) chain	460	150
	COL5A1 collagen type V alpha 1 (Fragment)	50	41

The average peptide length is calculated on peptides automatically annotated. Automatic annotation excludes very short peptides, since they cannot be univocally assigned to a specific proteins, thus the average peptide length is not corresponding to the one calculated by DH.

was found to be considerably lower than that previously observed at smaller scales, and 5.5 kg of unhydrolysed hides were recovered at the end of the reaction. The solution obtained had a protein concentration of 33 ± 2 mg/mL. Considering the final volume at the end of the reaction (34500 mL), total solubilised proteins (1140 ± 10 g) corresponded to ~ 40% of protein solubilisation. The kinetic profile of protein solubilisation was calculated on the average of protein content in the bovine hides (Figure 4).

This decrease in process efficiency was ascribed to two main issues; The first, as mentioned previously, was related to use of a different agitation system with lab-scale and semi-industrial equipment. In particular, in lab-scale experiments an agitator blade was used to ensure hides were covered completely by the buffer solution, (the enzyme was always in direct contact with hides). By contrast, in the semi-industrial plant an intermittent agitation drum was employed, and the hides were not always immersed in the solution. The second issue was likely caused by the lower rotation speed in the semi-industrial plant. Particularly, in lab-scale experiments a speed of 100 rpm was used, whereas in pilot plant speed was set at 10 rpm due to equipment limitations. The combination of these two factors undoubtedly resulted in a decreased hydrolytic efficiency. In order to improve this efficiency, another trial was performed under the same conditions, but with an increased hydrolysis time. The reaction was left overnight, corresponding to approx.16 hours hydrolysis. At the end of this second experiment, the hides were completely solubilised. The protein concentration obtained for the final solution was 71±4 mg/mL. Considering the final volume at the end of the reaction (40000 mL), total solubilised proteins (2839 ± 82 g) corresponded to a yield of 98% protein solubilisation. This experiment fully demonstrated the potential of the protocol to be applied on an industrial scale, achieving an almost complete protein solubilisation simply by prolonged reaction times. This is of course less desirable in terms of industrial costs, although in perspective other solutions could be devised to achieve the same efficiency. For instance, an additional solution may be to add equipment to grind the bovine hides and thus increase the contact surface, and consequently efficiency, of the process. However, in addition to the increased costs, the addition of this machinery would raise technical difficulties in grinding a fibrous material such as collagen. Alternatively, a reactor similar to that used at lab-scale could be employed. This reactor, equipped with an agitator blade, would result in a high process efficiency given the continuous contact between hides and the buffer at a higher agitation speed. This equipment may have the potential to enable complete hydrolysis with a reduced reaction time.

4. CONCLUSIONS

Our study demonstrated the industrial-scale feasibility of the recovery and valorisation of bovine hides, one of the relevant by-products of the meat industry, by means of enzymatic hydrolysis, providing high-quality hydrolysates at low cost and low environmental impact. These hydrolysates could be used as ingredients, additives or protein source for feed, food or other high-quality applications. Alcalase proved highly efficient in the digestion of these by-products using a high degree of hydrolysis, with large amounts of free amino acids released in solution. This process efficiency was strictly linked to the ability of Alcalase to degrade collagen, the main protein present in hides. Our results showed how Alcalase works efficiently at different hydromodules, underlining how ensuring a proper mixing and/or sufficient reaction time may result in the total solubilisation of proteins. Although complete optimization of all possible parameters was not undertaken, the selected conditions achieved a virtually complete protein solubilization at semi-industrial scale. Indeed, the semi-industrial pilot plant, an environmentally friendly process, is suitable for potential application at industrial scale, even achieving (with different equipment) higher efficiencies. It is noteworthy that prior to attaining an operational industrial process for the production of protein hydrolysates, the solutions

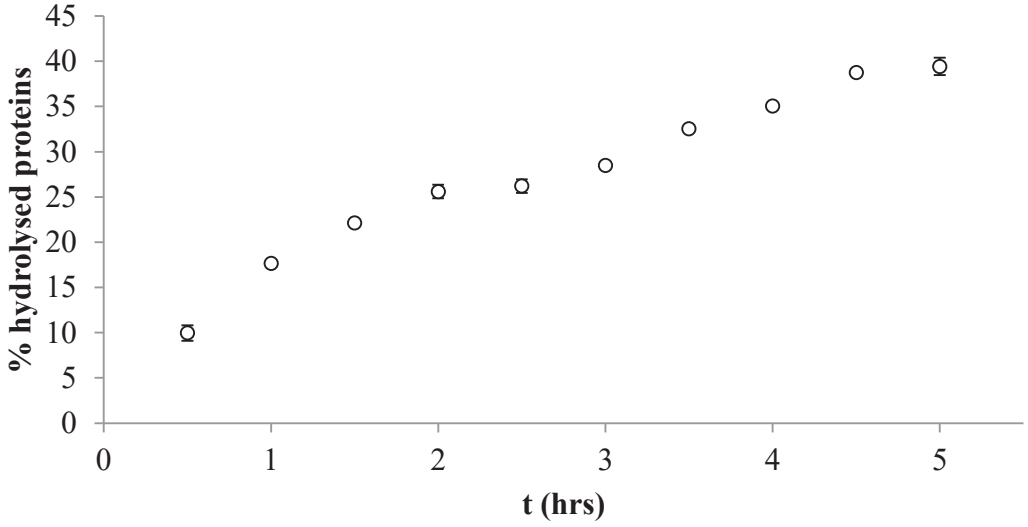

FIGURE 4: Kinetic profile of the percentage of the solubilised proteins over the total amount of proteins, as function of time in the pilot implant.

must be purified by removing undesirable components (ashes and fats), microbiologically hygienized, and finally water removed to obtain dry ingredients. The set up of these steps, which were beyond the scope of the present paper, in an economical and environmentally sustainable way, will pave the way to a product that can be sold on the market.

Taking into account an annual production of 7.3 million tonnes of bovines slaughtered in the EU alone (Eurostat data), the estimated amount of hides produced is in the region of half a million tonnes per year. Therefore, according to the data obtained in the present paper, if the hides are recovered using the proposed method, 150 ktonnes of proteins in form of soluble hydrolysates could be obtained. As a consequence, this method may contribute towards identifying a solution to the problem of disposal of recalcitrant by-products such as hides, whilst concomitantly decreasing the burden of environmental pollution and contributing towards the development of a new environmentally-friendly product.

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