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Enzymatic Hydrolysis of Meat Waste for a Circular Economy

Miguel Angulo, Mª Carmen Márquez *

Department of Chemical Engineering, Faculty of Chemical Sciences, University of Salamanca, Plaza de los Caídos 1-5, 37008-Salamanca, Spain; E-Mails: mianve20@usal.es; mcm@usal.es

* **Correspondence:** M. C. Márquez; E-Mail: mcm@usal.es

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Abstract

This work demonstrates the possibility of implementing a technology that allows profitable value to be drawn from the meat waste from retail stores. Protein hydrolysate, collagen and fatty acids were recovered from meat waste through enzymatic hydrolysis using a mixture of enzymes: a protease (Alcalase) and a lipase (Resinase). Enzymatic hydrolysis was studied by response surface methodology (RMS). Four independent variables were used to study the response variables. The analysis showed that all factors including protease/proteinic substrate ratio, lipase/lipidic substrate ratio, pH and temperature had a significant effect on responses of recovery of a protein hydrolysate, collagen, and fatty acids. From RSM-generated models, different optimum conditions were obtained depending on the product to be recovered. The economic study showed that operating profit depends on the operating conditions but that, in suitable conditions, it is four or more times higher than that obtained in the transformation of meat waste into meal for animal feed (the current destination of the meat waste that does not go to landfill). Consequently, the enzymatic treatment proposed for meat waste in this work is highly recommendable to maintain a circular economy for this biodegradable waste.

Keywords
Enzymatic hydrolysis; protein hydrolysate; collagen; fatty acids; response surface methodology; economic evaluation

1. Introduction

According to Food and Agricultural Organization (FAO) food losses relate to the reduction in edible food mass along the part of the supply chain that leads to edible food for human consumption [1]. Food losses come from the production, postharvest and processing phases in the food supply chain [2]. Food losses occurring at the final stage of the food chain (retail and final consumption) are named "food waste" [2].

Food waste (FW) is tackled in the EU Circular Economy Package, introducing a FW reduction target under the forthcoming Farm-to-Fork Strategy within the European Green Deal [3]. The countries of the EU are committed to halving per capita food waste at the retail and consumer stages by 2030 [4].

Meat has the highest wastage rates of any food during the retail period. About 70 percent of retail waste daily is produced by meat waste (MW) [5]. This large amount of waste should be reused for economic and ecological reasons.

Various methods of MW recovery have been used to avoid wasting by-products that can be obtained from them [6]. The current methods include composting as fertilizer [7] and production of meals for animals [8]. However, MW contains many essential nutrients such as proteins and lipids [5, 8] that permit additional value to this waste by generating innovative food and non-food products. Therefore, interest is increasing in studying these products to obtain additional value from the meat processing chain.

One possible way to process the waste material is using methodologies that do not affect its quality and properties. Enzymatic hydrolysis offers a fast and soft alternative to other chemical or mechanical treatments. The ability of the enzymes to hydrolyze proteins and lipids allows them to produce short peptides and release fatty acids.

Protein hydrolysates providing mainly di- and tripeptides are superior to whole proteins and free amino acids to be applied in several areas, such as biotechnology [9], nutrition [10] or cosmetics industries [11].

Collagen (an insoluble fibrous protein) is retail MW's major constituent of bones. The hydrolysis of collagen is more difficult than the hydrolysis of globular proteins in these wastes. The poor hydrolysis of collagen shows the advantage of allowing collagen recovery for commercial uses: cosmetics manufacturing, biomedical uses, etc. [12].

Fatty acids are long hydrocarbon chains with a carboxyl-terminal group. They are suitable for a wide range of applications in the energy domain, such as flow improvers for crude oils [13] and biofuels [14], or in other high-end industries such as food supplements [15], cosmetics [16], pharmaceutical applications [17] or surfactants [18].

Despite the increasing demand for protein hydrolysates, collagen and fatty acids, no studies have been conducted to use MW as a source for producing them. Therefore, the production of protein hydrolysates, collagen and fatty acids, by using enzymes as catalysts for hydrolysis, is an option to

generate more income for the MW from retail stores and to reach the target fixed in EU Circular Economy Package.

In this context, the present study aims twofold: i) to analyze the production of protein hydrolysates, collagen and fatty acids from MW, ii) to determine the operating profit of these products. To achieve these objectives, hydrolysis of MW was carried out at different pHs and temperatures with an enzymatic mixture consisting of a protease and a lipase.

2. Materials and Methods

2.1 Materials

MW was collected from a local retail meat store. The residue predominantly comprised flesh, bones, fat, heart, brain, liver, kidney, tongue, and lungs. Waste was ground to a size less than 1 mm, homogenized and frozen in small portions at 20°C. The portions of the frozen waste were thawed overnight in a refrigerator at 4°C before the hydrolysis.

The study employed two food-grade enzymes: Alcalase 2.4L and Resinase HT, kindly donated by Novozymes A/S (Bagsvaerd, Denmark). Alcalase hydrolyzes proteins to obtain protein hydrolysates and Resinase hydrolyzes lipids to obtain free fatty acids. Table S1 in the Supplementary material includes information about the characteristics of tested enzymes. These hydrolases were chosen for study based on their ability to produce high quantities of protein hydrolysate and fatty acids [19].

All chemicals used were of analytical grade.

2.2 Analysis Methods

MW and hydrolyzed samples were analyzed for moisture, proteinic substrate (proteins contained in MW), collagen, lipidic substrate (lipids contained in MW), fatty acids and saponification value according to the Association of Official Analytical Chemists (AOAC) methodology [20]. Moisture was determined by drying the sample 18 h in an oven at 102°C (method 950.46). Total N content was determined with the Kjeldahl procedure (method 981.10) and the protein content was subsequently converted from Kjeldahl N using a factor of 6.25 [21]. Collagen was quantified by hydroxyproline determination (method 990.26). Lipid content was determined by petroleum ether extraction (method 960.39). The fatty acids composition of the isolated lipids was analyzed by method 996.06; the procedure involves hydrolytic extraction, methylation, and capillary GC-FID analysis of the resulting fatty acid methyl esters (FAMEs). The saponification value was determined from the number of milligrams of potassium hydroxide required to neutralize the free fatty acid in the waste (method 920.160).

All measurements were made in triplicate and averaged. The results were expressed as mean ± standard deviation.

2.3 Protein and Lipids Hydrolysis

Hydrolysis was carried out in a well-stirred batch reactor (capacity 0.5 l) with magnetic stirring, temperature and pH control. 50 g of MW was added to a reaction vessel containing distilled water; in this way a concentration of proteinic substrate of 17.45 g/l and a concentration of lipidic substrate of 63.14 g/l were obtained in the reactor. The suspension was adjusted initially to the appropriate temperature and pH before adding the mixture of enzymes; pH and temperature were chosen

according to the optimal values of assayed enzymes (see Table S1 in Supplementary material). Once the enzymes were added, the pH of the reaction was constantly monitored; to keep the pH constant, the pH was adjusted to a desired value by adding 2 N NaOH every time the pH decreased 0.1 units from the desired value.

The resulting sample was boiled at 95-97°C for 20 minutes to deactivate the enzymes and to pasteurize the mixture and centrifuged at 9146 g for 15 minutes at room temperature to separate three phases: lower phase (unsolubilized waste) containing the collagen, intermediate phase (supernatant) containing the protein hydrolysate, and the upper phase containing the separated lipids comprising the free fatty acids.

All the tests were duplicated, and Student's t-test was performed to determine significant differences ($p < 0.05$).

The recovery of the products was determined as the percentage of total content in fresh MW according to the following equations:

$$Collagen\ recovery\ (\%) = \left[\frac{Collagen\ in\ the\ unsolubilized\ waste\ (g)}{Initial\ collagen\ in\ reactor\ (g)}\right] \times 100 \qquad (1)$$

$$Hydrolyzed\ protein\ recovery\ (\%) = \left[\frac{Protein\ in\ the\ supernatant\ (g)}{Initial\ protein\ in\ reactor\ (g)}\right] \times 100 \qquad (2)$$

$$Lipid\ recovery\ (\%) = \left[\frac{Lipids\ in\ upper\ phase\ after\ the\ centrifugation\ (g)}{Initial\ lipids\ in\ the\ reactor\ (g)}\right] \times 100 \qquad (3)$$

2.4 Mathematical Modelling and Optimization

Response surface modeling (RSM) was used to estimate the effect of operating parameters on MW recovery. For RSM, data include independent variables and response (dependent) variables. For this study, the independent variables included protease/proteinic substrate ratio (*Eo/So* = 0.11, 0.16, 0.21 UA/g), lipase/lipidic substrate ratio (*Eo´/So´* = 0.55, 0.83, 1.11 kLU/g), pH (*pH* = 7.5, 8.0, 8.5) and temperature (*T* = 45°C, 50°C, 55°C) while response variables included the percentage of recovered products such as percentage of solubilized protein (indicative of the amount of produced protein hydrolysate [22]), percentage of unhydrolyzed collagen (indicative of the amount of collagen that can be recovered) and percentage of recovered lipid (indicative of the amount of produced free fatty acids [23]). Values of independent variables were chosen according to previous studies [19]. The dependent variables were obtained from the experiments using Taguchi's L9 orthogonal array. The RSM modeling and optimization were carried out by Minitab 19.

The RSM model was developed using a quadratic equation with interaction terms between independent variables. The model was obtained for each response variable and tested using ANOVA for significance. The response equations were graphically demonstrated using contour plots. Response equations were used to optimize the dependent variables: to maximize the percentage of recovered products.

2.5 Economic Analysis

The operating profit (R) of the process of transformation of MW into protein hydrolysate, collagen and free fatty acids has been calculated as the total income generated from sales (I_T) after paying off processing costs (C_T):

$$R = I_T - C_T \tag{4}$$

2.5.1 Processing Costs

The processing costs can be divided into two stages: stage of reaction (C_{RS}) and stage of product separation and purification (C_{SS}). The pretreatment costs (corresponding to the stages of milling and sieving before the MW is fed into the reactor) and the operating labour costs have not been included because they are negligible.

$$C_T = C_{RS} + C_{SS} \tag{5}$$

C_{RS} is calculated from the sum of the cost of reagents and catalysts (C_R) and the calorific cost (C_C) involved in the hydrolysis [24]:

$$C_{RS} = C_R + C_C \tag{6}$$

2.5.2 Total Income Generated from Sales

The total income (I_T) was calculated from the sales of protein hydrolysate (I_H), unhydrolyzed collagen (I_C) and free fatty acids (I_A):

$$I_T = I_H + I_C + I_A \tag{7}$$

3. Results and Discussion

3.1 Meat Waste Characterization

MW had a moisture of 45.04 ± 1.21% and a protein and lipid content of 19.03 ± 0.42% and 68.91 ± 1.14% (both on a dry weight basis), respectively. Although there are only a few references to MW, the paper of García et al. [5] supports our results; they carried out a study on the characterization of MW generated in 208 butchers finding, on a dry weight basis, a 24.6 ± 10.3% in protein content and a 69.9 ± 13.7% in lipid content.

Among the proteins, collagen has been specifically determined in waste. The collagen amount was found to be 67.91 ± 0.14 mg/g. This amount represents 35.68% of the total protein present in waste which is consistent with the fact that collagen is the most abundant protein in the mammal´s body, representing 25% of the total body protein and 95% of the fibrous elements of connective tissue [25] and 90% of the bones [26]. Considering that connective tissue and bones in MW are in a higher proportion than usual in animal bodies, it is not surprising that the obtained percentage of collagen slightly exceeds the usual 25% in body protein.

The fatty acid composition of MW (Table 1) reveals the dominance of unsaturated fatty acids (53.36 ± 0.33) as compared to saturated fatty acids (46.64 ± 0.19). Since there are no data in the

literature regarding the fatty acid content in butcher waste, a bibliographic review was done on the fatty acid content in different animal meats and fats [19]. The review observed that fatty acid content varies greatly depending on whether the sample is flesh or lard and on the origin of the sample (beef, lamb, rabbit, chicken…). With all data obtained, a range of values was determined; the results are included in Table 1. It corroborated well with our report on fatty acids because the percentages obtained for each fatty acid in waste are within the specified range of bibliographic values.

Table 1 Fatty acids profile in lipids from MW.

Fatty acid	MW Mean ± SD	Bibliographic values Minimum-Maximum
C14:0	4.25 ± 0.09	1.70-32.9
C15:0	0.27 ± 0.03	0.00-0.60
C16:0	25.45 ± 0.26	2.28-36.89
C17:0	3.48 ± 0.10	0.00-3.90
C18:0	12.85 ± 0.24	0.40-33.80
C20:0	0.34 ± 0.02	0.06-4.69
C14:1	0.00 ± 0.00	0.00-2.38
C15:1	0.00 ± 0.00	0.00-1.70
C16:1	0.30 ± 0.01	0.00-37.9
C17:1	0.32 ± 0.02	0.00-0.90
C18:1n9c+t	24.64 ± 0.34	0.84-46.29
C20:1	0.00 ± 0.00	0.00-2.78
C18:2n6c+t	10.50 ± 0.27	0.49-38.28
C20:2	1.76 ± 0.05	0.00-2.57
C18:3	3.31 ± 0.06	0.25-4.71
C20:3n3	4.86 ± 0.05	0.07-4.82
C20:3n6+c21:0	3.88 ± 0.08	0.07-4.82
C20:4n6	3.78 ± 0.07	0.03-3.83

Saponification value for MW was 200.21 ± 1.94. This value agrees with the literature data where values around 200 are given for saponification value of animal fats [27].

3.2 Response Surface Modelling

The independent and dependent variables were analyzed to obtain a regression equation that could predict the response within the given range. Based on the experimental results obtained from the Taguchi's L9 orthogonal array (Table 2), a response surface model was developed for the recovered products.

Table 2 Taguchi L9 experiment design and final products of MW hydrolysis.

No	Eo/So (UA/g)	Eo´/So´ (kLU/g)	pH	T (°C)	Hydrolyzed protein recovery (%)	Collagen recovery (%)	Lipid recovery (%)
1	0.11	0.55	7.5	45	52.61 ± 0.07	58.66 ± 0.48	85.96 ± 0.17
2	0.11	0.83	8.0	50	49.85 ± 0.26	61.28 ± 0.04	86.44 ± 1.78
3	0.11	1.11	8.5	55	53.31 ± 0.03	55.58 ± 0.09	91.26 ± 0.04
4	0.16	0.55	8.0	55	50.60 ± 0.01	60.25 ± 0.14	91.24 ± 0.17
5	0.16	0.83	8.5	45	58.90 ± 0.25	50.80 ± 0.09	94.45 ± 0.29
6	0.16	1.11	7.5	50	67.26 ± 0.11	46.30 ± 0.02	97.02 ± 0.25
7	0.21	0.55	8.5	50	64.53 ± 0.10	47.27 ± 0.01	95.15 ± 0.28
8	0.21	0.83	7.5	55	66.14 ± 0.13	46.68 ± 0.04	94.97 ± 0.12
9	0.21	1.11	8.0	45	69.31 ± 0.26	41.83 ± 0.20	97.17 ± 0.07

Contour plots in Figure 1 show the interaction between two independent variables keeping the 3rd and the 4th variables at the central point for the percentage of hydrolyzed protein. Similar figures were obtained for the other dependent variables. The obtained quadratic model equations for the different recovered products after hydrolysis are:

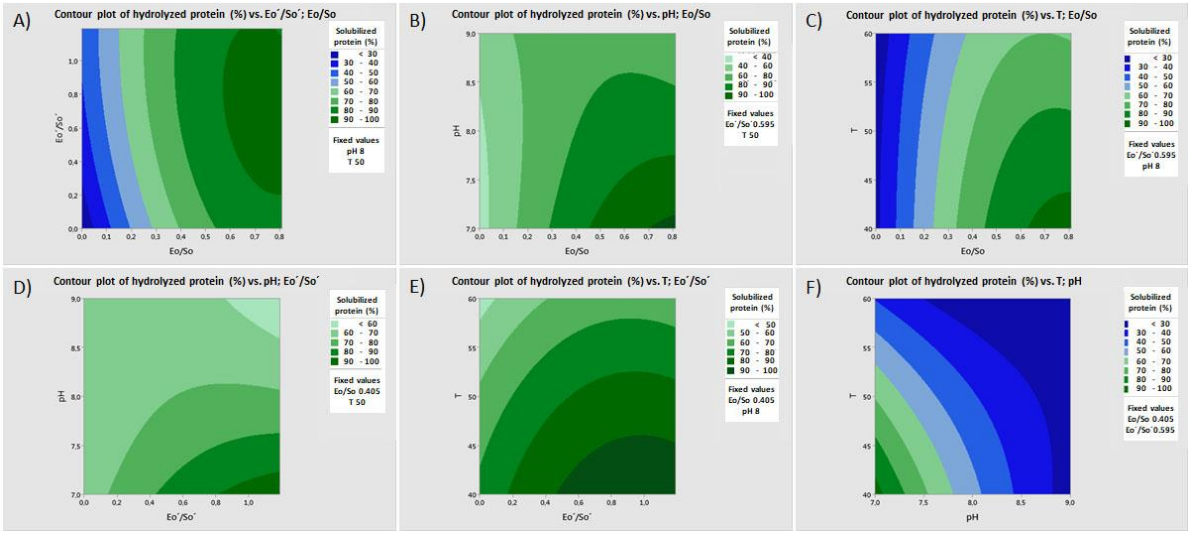

Figure 1 Contour plots for the influence of the independent variables on the percentage of hydrolyzed protein: (A) *Eo´/So´* × *Eo/So*; (B) pH × *Eo/So*; (C) temperature × *Eo/So*; (D) pH × *Eo´/So´*; (E) temperature × *Eo´/So´*; and (F) temperature × pH.

$$\text{Hydrolyzed protein recovery (\%)} = 326.00 + 584.00 \cdot \text{Eo/So} + 183.10 \cdot \text{Eo´/So´} - 69.20 \cdot \text{pH} - 3.85 \cdot \text{T} - 97.40 \cdot \text{Eo/So} \cdot \text{Eo/So} - 12.17 \cdot \text{Eo´/So´} \cdot \text{Eo´/So´} + 3.5 \cdot \text{pH} \cdot \text{pH} - 0.01 \cdot \text{T} \cdot \text{T} - 16.9 \cdot \text{Eo/So} \cdot \text{Eo´/So´} - 45.20 \cdot \text{Eo/So} \cdot \text{pH} - 1.28 \cdot \text{Eo/So} \cdot \text{T} - 18.30 \cdot \text{Eo´/So´} \cdot \text{pH} - 0.13 \cdot \text{Eo´/So´} \cdot \text{T} + 0.63 \cdot \text{pH} \cdot \text{T} \quad (8)$$

$$\text{Lipid recovery (\%)} = -348.00 + 957.00 \cdot \text{Eo/So} + 222.20 \cdot \text{Eo´/So´} + 69.90 \cdot \text{pH} + 1.71 \cdot \text{T} - 322.60 \cdot \text{Eo/So} \cdot \text{Eo/So} - 31.36 \cdot \text{Eo´/So´} \cdot \text{Eo´/So´} - 2.39 \cdot \text{pH} \cdot \text{pH} - 0.02 \cdot \text{T} \cdot \text{T} - 303.40 \cdot \text{Eo/So} \cdot \text{Eo´/So´} - 90.40 \cdot \text{Eo/So} \cdot \text{pH} + 2.15 \cdot \text{Eo/So} \cdot \text{T} - 17.55 \cdot \text{Eo´/So´} \cdot \text{pH} + 0.23 \cdot \text{Eo´/So´} \cdot \text{T} - 0.1 \cdot \text{pH} \cdot \text{T} \quad (9)$$

$$\begin{aligned}\text{Collagen recovery (\%)} = {} & -562.00 - 356.00 \cdot \text{Eo/So} + 20.30 \cdot \text{Eo´/So´} + \\ & 83.30 \cdot \text{pH} + 12.69 \cdot \text{T} + 248.80 \cdot \text{Eo/So} \cdot \text{Eo/So} - 22.87 \cdot \text{Eo´/So´} \cdot \text{Eo´/So´} - \\ & 1.64 \cdot \text{pH} \cdot \text{pH} - 0.02 \cdot \text{T} \cdot \text{T} - 33.60 \cdot \text{Eo/So} \cdot \text{Eo´/So´} + 4.60 \cdot \text{Eo/So} \cdot \text{pH} + \\ & 1.03 \cdot \text{Eo/So} \cdot \text{T} + 5.46 \cdot \text{Eo´/So´} \cdot \text{pH} - 0.56 \cdot \text{Eo´/So´} \cdot \text{T} - 1.24 \cdot \text{pH} \cdot \text{T} \end{aligned} \quad (10)$$

ANOVA analysis was conducted to test the significance of developed model equations for the response variables (Table 3). The ANOVA results show that all the response variables have a significant correlation (P < 0.0001). The model has provided a good fit with the experimental data since the coefficients of determination R^2 had values higher than 0.95 (Table 3). This means that the fitted model could explain more than 95% of the total variability within the range of values studied. According to Sin et al. [28], values of R^2 higher than 0.80 indicates a good relevance of the dependent variables in the model, and the model fits well with the actual data when R^2 approaches unity.

Table 3 ANOVA analysis of the response model equations (8) – (10) for response variables.

Response variable (%)	DF	F-value	P-value	R^2	Adjusted R^2	Remarks
Hydrolyzed protein recovery	14	44.89	<0.0001	0.9593	0.9573	Significant
Lipid recovery	14	15.09	<0.0001	0.9830	0.9245	Significant
Collagen recovery	14	254.72	<0.0001	0.9922	0.9883	Significant

However, a large value of R^2 does not always imply that the model is good. Adjusted R^2 is a modified R^2 that adjusts for the number of explanatory terms in a model. The adjusted R^2 increases only if the new term improves the model more than expected by chance [29]. For a good statistical model, the adjusted R^2 should be higher than 0.80, while the difference between R^2 and adjusted R^2 must be less than 0.2 [30]. Both requirements were met in this case (Table 3). Hence, the model equations can predict the percentage of recovered products based on *Eo/So*, *Eo´/So´*, pH, and temperature.

The model's usefulness was further validated using numerous previous experiments with the same enzymes used separately [19]. Table S2 (Supplementary material) shows the data from the validation experiment. The observed and predicted values, from the experimental runs and validation runs, were compared to evaluate the validity of the above model (Figure 2). The regression coefficients and slopes of regression equations for observed versus predicted values were close to 1.0. These high correlation values confirm the prediction model's validity inside and outside the given range of the independent variables analyzed in this paper.

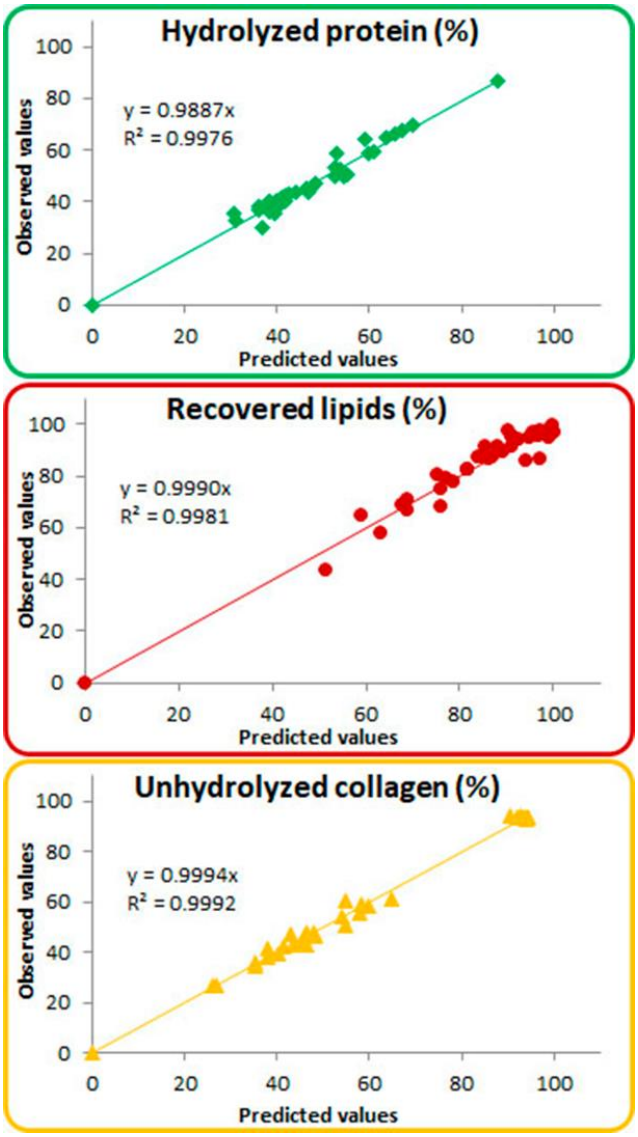

Figure 2 Parity plots of predicted values versus actual values.

The response optimizer of Minitab 19 was used to identify the appropriate combination of the hydrolysis variables to obtain the optimal value of the interesting responses. The objective was to maximize the response for the recovered products, trying to achieve targets of 100%.

The 100% hydrolyzed protein was achieved at Eo/So = 0.65 UA/g, Eo´/So´ = 0.51 kLU/g, pH = 7.50 and temperature = 52.00°C; under these conditions, 14.74% of collagen and 80.71% of lipids would be recovered. The recovered lipids were found to be maximum (100%) at optimum parameters of Eo/So = 0.16 UA/g, Eo´/So´ = 0.83 kLU/g, pH = 8.00 and temperature = 46.66°C where the recovery of the other products would be 61.98% of hydrolyzed protein and 52.64% of collagen. As expected, the optimizer indicated that the maximum level of recovered collagen (100%) could be achieved only in the absence of protease with a hydrolysis temperature of 55.00°C, a pH of 7.00 and a lipase/lipidic substrate ratio of 0.60 kLU/g; then, 28.47% of hydrolyzed protein and 56.82% of lipids would be recovered.

As can be seen, different combinations of the hydrolysis variables were obtained depending on the desired recovery target. pH and temperature for optimization were within a narrow range of values: 7-8 for pH and 47-57°C for temperature.

The maximization of hydrolyzed protein is favored by the presence of Resinase in the reactor. The cleavage of triglyceride bonds by lipases produces the release of the proteins in the MW; thus, peptide bonds get exposed and are available for cleavage by proteases [19].

The maximization of recovered lipids is favored by the presence of Alcalase in the reactor. From a chemical point of view, proteases cleave the peptide bonds which produces the release of lipids contained in the MW; the greater the peptide bond cleavage, the greater amount of lipids released [31].

The maximization of recovered collagen implies no protease in the reactor. Although collagen is a fibrous protein difficult to hydrolyze with water alone, a greater or lesser part can be hydrolyzed in the presence of proteases [32].

In order to achieve a circular economy, it is not enough to recover the products. However, the recovery cost would also be lower than the value of the recovered materials. Therefore, an economic evaluation of the process proposed in this study must be carried out to verify its usefulness in a circular economy.

3.3 Economic Evaluation

The results obtained in our laboratory experiments have been used to estimate the operating profit involved in the treatment of 1 kg of MW.

3.3.1 Cost of Reagents and Catalysts (C_R)

Besides waste and water, enzymes as catalysts and NaOH as pH controller are necessary for hydrolysis. Waste, NaOH and water costs are insignificant compared to enzymes. Then the cost of the item of reagents and catalysts can be expressed as follows:

$$C_R = a_P \cdot p_P + a_L \cdot p_L \tag{11}$$

where a_P is the activity of the protease used in the treatment, p_P is the price per unit of protease activity ($1.46 \cdot 10^{-2}$ €/UA), a_L is the activity of the lipase used in the treatment and p_L is the price per unit of lipase activity ($0.70 \cdot 10^{-3}$ €/kLU). Prices were given by the enzymes supplier.

Enzymatic activity was determined as function of enzyme initial concentration and reactor volume:

$$a_P = E_o \cdot V \tag{12}$$

$$a_L = E_o^{'} \cdot V \tag{13}$$

where *Eo* is the initial concentration of Alcalase (UA/l), *Eo´* is the initial concentration of Resinase (kLU/l) and *V* is the mixture volume in the reactor.

The mixture volume in the reactor (*V* = 6.0 l) was obtained from the ratio between 1 kg (1000 g) of MW and the initial concentration of MW in experiments (166.67 g/l).

By the above, it is obvious that the cost of this item depends on the initial concentration of enzymes but also the initial concentration of MW (Table 4).

Table 4 Heats and costs of the stages of reaction and separation involved in the process.

No	*T* (°C)	*Eo* (UA/l)	*Eo´* (kLU/l)	S_f *(g/l)*	S_f´ *(g/l)*	a_P (UA)	a_L (kLU)	Q_{R1} (kJ/kg$_{MW}$)	Q_{R2} (kJ/kg$_{MW}$)	Q_R (kJ/kg$_{MW}$)	Q_P (kJ/kg$_{MW}$)	Q_m (kJ/kg$_{MW}$)	Q_c (kJ/kg$_{MW}$)	Q (kJ/kg$_{MW}$)	C_R (€/kg$_{MW}$)	C_C (€/kg$_{MW}$)	C_{RS} (€/kg$_{MW}$)	C_{SS} (€/kg$_{MW}$)
1	45	1.87	34.95	5.35	14.35	11.22	209.70	-1.86	2.00	0.15	596.61	596.76	646.42	1,298.84	0.311	0.019	0.330	1.320
2	50	1.87	52.44	5.16	14.45	11.22	314.63	-1.79	2.02	0.23	715.94	716.17	775.70	1,557.40	0.384	0.022	0.406	1.624
3	55	1.87	69.89	5.40	15.45	11.22	419.33	-1.87	2.16	0.29	835.26	835.55	904.98	1,815.96	0.457	0.026	0.483	1.932
4	55	2.81	34.95	5.22	15.45	16.86	209.70	-1.81	2.16	0.35	835.26	835.61	904.98	1,815.96	0.393	0.026	0.419	1.676
5	45	2.81	52.44	5.75	16.08	16.86	314.63	-1.99	2.25	0.25	596.61	596.86	646.42	1,298.84	0.466	0.019	0.485	1.940
6	50	2.81	69.89	6.23	16.58	16.86	419.33	-2.16	2.32	0.16	715.94	716.10	775.70	1,557.40	0.540	0.022	0.562	2.248
7	50	3.74	34.95	6.08	16.22	22.44	209.70	-2.11	2.27	0.16	715.94	716.10	775.70	1,557.40	0.474	0.022	0.496	1.984
8	55	3.74	52.44	6.17	16.19	22.44	314.63	-2.14	2.26	0.12	835.26	835.38	904.98	1,815.96	0.548	0.026	0.574	2.296
9	45	3.74	69.89	6.33	16.61	22.44	419.33	-2.19	2.32	0.13	596.61	596.74	646.42	1,298.84	0.621	0.019	0.640	2.560

3.3.2 Calorific Cost (C_C)

Since the process is carried out at temperatures higher than room temperature, the calorific cost includes the cost of heating the reaction mixture until the reaction temperature is reached and the cost of keeping this temperature during the hydrolysis time. Fuel consumption was calculated from the ratio between the heat required in the process (Q) and the net calorific value of the fuel (NCV). Calorific cost is the product of fuel consumption and fuel price (p_C):

$$C_C = \frac{Q}{NCV} \cdot p_C \tag{14}$$

Natural gas (NCV = 47,087 kJ/kg and p_C = 0.676 €/kg) is proposed as fuel. Data of the natural gas was given by the gas supplier.

As stated above, the heat involved in the hydrolysis (Q) is divided in two terms: one corresponding to the heating of the reaction mixture (Q_C) and the other one corresponding to keep the hydrolysis temperature (Q_m):

$$Q = Q_C + Q_m \tag{15}$$

Q_C can be estimated from the following equation:

$$Q_C = m_c \cdot c_p \cdot (T - T_o) \tag{16}$$

where m_c is the mass of reaction mixture, c_p is its heat capacity, T is the reaction temperature and T_o is the initial inlet temperature of the mixture *(*20°C*)*.

The mass of the reaction mixture (m_c = 6,18 kg) was calculated from the mixture volume in the reactor and the mixture density; for operating conditions used in this work, the mixture density was 1.03 g/ml. Because the MW concentration is relatively low, the mixture heat capacity was considered equal to that of the water: c_p = 4,186 kJ/kg·°C [33].

Q_m was calculated as the sum of the external heat necessary to compensate heat loss if the reactor was not jacketed (Q_P) and the heat of the reactions (Q_R):

$$Q_m = Q_P + Q_R \tag{17}$$

Q_p can be estimated from the overall coefficient of heat transfer (U), the heat transfer area (A_t), the reaction time (t), the reaction temperature (T) and the room temperature (T_{room} = 20°C) according to the expression [34]:

$$Q_p = U \cdot A_t \cdot t \cdot (T - T_{room}) \tag{18}$$

The value of the overall coefficient of heat transfer (U) was considered as 10.2 W/m^2·°C, a typical value given in the literature for vessels like the one used in this work [34]. The reaction time was set at 240 min.

The heat transfer area (A_t = 0.162 m^2) was determined from the volume necessary to treat 1 kg of MW in a cylindrical reactor partially filled (75%) with the reaction mixture and whose diameter is equal to the liquid height [35]:

$$A_t = \frac{{}^{16}/_{3} \cdot V}{\left({}^{4 \cdot V}/_{\pi}\right)^{1/3}} \tag{19}$$

In a jacket cooling system, the area available for heat transfer is limited to the external surface of the tank, without considering the top nor the bottom where the piping system should be installed.

Q_R consists of two parts: heat for peptide bonds hydrolysis (Q_{R1}) and heat for ester bond hydrolysis (Q_{R2}):

$$Q_R = Q_{R1} + Q_{R2} \tag{20}$$

The heats of hydrolytic enzymatic reactions (Q_{R1} and Q_{R2}) were calculated as follow:

$$Q_{R1} = \Delta H_{R1} \cdot \left(S_o - S_f\right) \cdot h_T \cdot V \tag{21}$$

$$Q_{R2} = \Delta H_{R2} \cdot (S_o^{'} - S_f^{'}) \cdot \frac{1}{MM_{AG}} \cdot V \tag{22}$$

where ΔH_{R1} is the average enthalpy for peptide bond hydrolysis, ΔH_{R2} is the average enthalpy for ester bond hydrolysis, So is the initial concentration of proteinic substrate in the reactor (17.45 g/l), $So´$ is the initial concentration of lipidic substrate in the reactor (63.24 g/l), S_f is the final concentration of proteinic substrate in the reactor, $S_f´$ is the final concentration of lipidic substrate in the reactor, h_T is the total peptide bonds in the proteinic substrate of MW (7.6 mol/kg for animal protein [21]), MM_{AG} is the average molar mass of fatty acids in the lipidic substrate of MW, and V is the mixture volume in the reactor. According to bibliography ΔH_{R1} = -7.6 ± 2.0 kJ/mol and ΔH_{R2} = 1.8 ± 0.2 kJ/mol [36].

Given that the saponification value is inversely proportional to average molar mass of a lipid [37], the relationship between the average molar mass of a lipid and the average molar mass of the fatty acids contained in it is 3 [38], and one mole of lipid react with three moles of KOH (168000 mg of KOH) in a saponification reaction [39], MM_{AG} of 279.73 Daltons was calculated from the following equation (23):

$$MM_{AG} = \frac{56.000}{Saponification\ value} \tag{23}$$

The values of the different heats involved in the process and the calorific cost are included in the Table 4.

3.3.3 Reaction Cost (C_{RS})

Table 4 shows the cost of the stage of the reaction. From the results obtained, it can be deduced that the operating cost of the enzymatic reactor is mainly conditioned by the cost of the enzymes, representing around 95% of the total cost of the reaction stage in the studied experiments.

3.3.4 Product Separation and Purification Cost (C_{SS})

As in this work the separation and purification of the products have not been carried out, the corresponding cost was estimated from the literature where the cost of downstream processing of products obtained in a bioreactor represents 60-80% of the processing cost [40-44]. According to a conservative estimation, the separation and purification costs were calculated as 80% of the processing cost and then:

$$C_{SS} = \frac{80}{20} C_{RS} \tag{24}$$

Table 4 includes values of product separation and purification cost for the carried out experiments.

3.3.5 Total Processing Costs

To obtain the processing cost, the cost of the stage of reaction (C_{RS}) was added to the cost of the product separation and purification (C_{SS}). Table 6 shows the obtained values. These values indicate that processing cost ranges between €1.65 to €3.20 per kg of processed MW for the analyzed operating conditions.

3.3.6 Income Generated from Sales

The income from the sale of the products is calculated from the amount of each product obtained and their sale price given by suppliers:

$$I_H = m_H \cdot p_H \tag{25}$$

$$I_C = m_C \cdot p_C \tag{26}$$

$$I_A = \sum m_A \cdot p_A \tag{27}$$

where I_H is the income generated from sale of protein hydrolysate, I_C is the income generated from sale of unhydrolyzed collagen, I_A is the income generated from sale of free fatty acids, m_H is the amount of the obtained protein hydrolysate that is equivalent to the amount of the solubilized protein, m_C is the amount of the obtained unhydrolyzed collagen, m_A is the amount of the obtained free fatty acids, p_H is the price of the protein hydrolysate, p_C is the price of the collagen, and p_A is the price of the free fatty acids.

Table 5 shows prices and amounts of the protein hydrolysates and the unhydrolyzed collagen obtained in the experiments.

Table 5 Prices and amounts of protein hydrolysate, unhydrolyzed collagen and fatty acids obtained in different experiments.

Product	Price (€/kg)	Amount of product (kg_{produt}/kg_{MW})								
		No								
		1	2	3	4	5	6	7	8	9
Protein hydrolysate	25.52	0.055	0.052	0.056	0.053	0.062	0.070	0.068	0.069	0.073
Unhydrolyzed collagen	24.15	0.022	0.023	0.021	0.023	0.019	0.017	0.018	0.017	0.016
C14:0	4.52	7.90E-03	8.05E-03	9.19E-03	8.85E-03	9.96E-03	1.06E-02	1.01E-02	1.00E-02	1.04E-02
C15:0	3.60	3.41E-04	3.51E-04	3.91E-04	3.91E-04	4.24E-04	4.50E-04	4.37E-04	4.25E-04	4.46E-04
C16:0	6.01	5.45E-02	5.44E-02	6.71E-02	6.56E-02	7.27E-02	7.78E-02	7.63E-02	7.19E-02	7.97E-02
C16:1	4.85	2.56E-04	2.61E-04	3.04E-04	3.05E-04	3.38E-04	3.58E-04	3.43E-04	3.42E-04	3.58E-04
C17:0	3.69	4.71E-03	5.09E-03	5.78E-03	5.68E-03	6.32E-03	6.61E-03	6.26E-03	6.35E-03	6.65E-03
C17:1	4.94	1.07E-03	1.14E-03	1.26E-03	1.27E-03	1.45E-03	1.53E-03	1.45E-03	1.47E-03	1.55E-03
C18:0	5.57	3.86E-02	3.99E-02	4.33E-02	4.25E-02	4.65E-02	4.79E-02	4.66E-02	4.65E-02	4.90E-02
C18:1	4.13	6.76E-02	6.97E-02	7.47E-02	7.58E-02	7.83E-02	8.45E-02	7.77E-02	7.82E-02	8.74E-02
C18:2	13.92	3.76E-02	3.83E-02	4.26E-02	4.29E-02	4.46E-02	4.90E-02	4.77E-02	4.64E-02	4.90E-02
C18:3n3	4.94	9.54E-03	1.04E-02	1.17E-02	1.17E-02	1.29E-02	1.38E-02	1.31E-02	1.31E-02	1.38E-02
C18:3n6	4.94	1.34E-04	1.22E-04	1.56E-04	1.59E-04	1.65E-04	1.84E-04	1.81E-04	1.73E-04	1.84E-04
C20:0	4.04	9.01E-04	9.20E-04	1.03E-03	1.03E-03	1.12E-03	1.18E-03	1.13E-03	1.07E-03	1.18E-03
C20:2	5.39	8.11E-03	8.34E-03	9.18E-03	9.18E-03	9.75E-03	1.03E-02	9.87E-03	9.82E-03	1.04E-02
C20:3n3	6.08	8.32E-03	8.59E-03	9.27E-03	9.12E-03	9.86E-03	1.02E-02	1.01E-02	9.80E-03	1.04E-02
C20:3n6	6.08	5.04E-03	5.12E-03	6.02E-03	5.94E-03	7.00E-03	7.48E-03	6.92E-03	6.46E-03	7.36E-03
C20:4n6	5.21	4.73E-03	5.11E-03	5.99E-03	6.02E-03	6.57E-03	7.58E-03	6.96E-03	6.66E-03	7.65E-03

The upper phase containing the separated lipids was analyzed for free fatty acids to determine the value of the income generated from the sale of free fatty acids. Prices and amounts of free fatty acids obtained in the experiments are given in Table 5.

Income generated from sales (Table 6) were obtained from data of Table 5.

Table 6 Incomes generated from sales, total processing costs and operating profit for different experiments.

No	*Eo/So* (AU/g)	*Eo´/So´* (kLU/g)	*pH*	*T* (°C)	I_H (€/kg$_{MW}$)	I_C (€/kg$_{MW}$)	I_A (€/kg$_{MW}$)	I_T (€/kg$_{MW}$)	C_T (€/kg$_{MW}$)	*R* (€/kg$_{MW}$)
1	0.11	0.55	7.5	45	1.406	0.529	1.607	3.542	1.650	1.892
2	0.11	0.83	8.0	50	1.332	0.553	1.644	3.529	2.030	1.499
3	0.11	1.11	8.5	55	1.424	0.501	1.854	3.779	2.415	1.364
4	0.16	0.55	8.0	55	1.352	0.544	1.847	3.743	2.095	1.648
5	0.16	0.83	8.5	45	1.574	0.458	1.987	4.019	2.425	1.594
6	0.16	1.11	7.5	50	1.797	0.418	2.125	4.340	2.810	1.530
7	0.21	0.55	8.5	50	1.724	0.426	2.046	4.196	2.480	1.716
8	0.21	0.83	7.5	55	1.767	0.421	1.995	4.183	2.870	1.313
9	0.21	1.11	8.0	45	1.852	0.377	2.155	4.384	3.200	1.184

3.3.7 Operating Profit

The operating profit has been calculated by subtracting the processing costs (C_T) from the total income generated from sales (I_T) (Table 6).

For the operating conditions investigated in this study, the experiment carried out at a lowest *Eo/So*, *Eo´/So´*, pH and temperature was the most profitable treatment.

Operating profits obtained from the results of optimization agree with this conclusion. According to the optimization study carried out in section 3.2, *Eo/So*, *Eo´/So´* and temperature have high values for 100% of hydrolyzed protein (*Eo/So* = 0.65 UA/g, *Eo´/So´* = 0.51 kLU/g, *T* = 52.00°C) so there are no profits but only economic losses: *R* = -1.606 €/kg$_{MW}$. However, these values are not high for 100% of recovered lipids (*Eo/So* = 0.16 UA/g, *Eo´/So´* = 0.83 kLU/g, *T* = 46.66°C) and, in consequence, the profit is very good: *R* = 1.985 €/kg$_{MW}$. In the case of 100% of unhydrolyzed collagen, although *Eo/So* = 0 UA/g and *Eo´/So´* = 0.60 kLU/g, the high hydrolysis temperature value (*T* = 55.00°C) and the low amount of collagen compared to that of protein or lipids in MW cause a decrease in the profit: R = 0.951 €/kg$_{MW}$.

As seen from the results, for the appropriate operating conditions, operating profits ranged from €1 to €2 per kg of treated MW. To know if this value recommends or advises against the use of MW in a circular economy, the profit obtained from hydrolysis was compared with the profit currently obtained from the sale of MW.

At present, the destination of the MW that does not go to the landfill is its transformation into flour for animal feed. The sale price of meat and bone meal is €0.5/kg; since the meal contains exclusively meat and bone, that is, the meal is 100% MW, the price can be expressed as €0.5/kg of treated MW. The processing costs of meal production from MW are 50% of the sale price [45]. Therefore, the operating profit of MW meal is about €0.25/kg of treated MW.

According to this, operating profits obtained in the transformation of MW into protein hydrolysates, collagen and fatty acids would be, in the worst case of the appropriate operating conditions, four times higher than that obtained in the transformation of MW into a meal. This value is eight times in the best operating condition (100% recovered lipids).

The high profitability of this treatment suggests that it could be adapted to many shops shortly.

4. Conclusions

Executed research showed the possibility of recycling MW from retail shops producing valuable products: protein hydrolyzed, collagen and fatty acids.

RMS studied enzymatic hydrolysis of protein and lipids from MW. A mixture of Alcalase and Resinase was used for the hydrolysis and four independent variables were used to study the response variables: *Eo/So*, *Eo´/So´*, pH and temperature. The analysis of observed values by ANOVA indicated that all four variables and the interaction between them significantly affected the products recoveries. These could be related to the enzymatic hydrolysis conditions by quadratic model equations with interaction terms between independent variables.

The usefulness of the obtained prediction models was further validated using different combinations (other than used for optimization) of four factors and comparing the observed and predicted values. The values of the regression coefficients and the slope of the regression equations close to 1.0 indicate the validity of the prediction models.

The appropriate combination of the hydrolysis variables to maximize the response for the recovered products (targets of 100% of hydrolyzed protein, 100% of recovered collagen or 100% of recovered lipids) were analyzed; different combinations of the hydrolysis variables were obtained depending on the desired recovery target.

The economic evaluation was conducted to determine the operating profit in treating MW. Processing costs and income from sales of hydrolyzed protein, collagen and fatty acids obtained by the enzymatic hydrolysis were calculated. The operating conditions investigated in this study showed that the experiments performed at the lowest *Eo/So*, *Eo´/So´*, pH and temperature were the most profitable treatments. In the suitable conditions, the operating profits obtained ranged from €1 to €2 per kg of treated MW and were from four to eight times higher than that obtained in the transformation of MW into meal for animal feed that is the current destination of the MW that does not go to landfill. According to these results, the optimum hydrolysis conditions to obtain the maximum profit were *Eo/So* = 0.16 UA/g, *Eo´/So´* = 0.83 kLU/g, *pH* = 8.00 and *T* = 46.66°C *(*100% of recovered lipids*)*.

Consequently, the enzymatic treatment proposed for MW in this work is very appealing to keep a circular economy system that enables reuse of resources without having them end up at a landfill.

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Author Contributions

M. Angulo: Methodology, Software, Validation, Investigation. **M.C. Márquez:** Conceptualization, Formal analysis, Data curation, Writing – original draft, Supervision, Project administration, Funding acquisition.

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Competing Interests

The authors have declared that no competing interests exist.

Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Table S1: Characteristics of tested enzymes.
2. Table S2: Values observed during validation experiments along with corresponding predicted values under different combinations of independent variables.

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