Influence of the Extraction Temperature on the Properties of Biopolymers Obtained from Tannery Wastes

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ABSTRACT: The tanning industry generates very large quantities of industrial wastes. The advancement of European policy and legislation protecting the environment has prompted the transformation of tannery solid waste materials into valuable co-products, useful to be recycled or employed in other industries. The objective of this work is to obtain gelatine from tannery wastes, in order to reuse it as natural microencapsulating agent in the production of active materials with functional properties. Concretely, this paper focuses on the influence of the extraction temperature on gelatine properties and its microencapsulating ability. An alternative enzymatic pre-treatment to the conventional alkaline one is proposed in order to save costs and reduce time, as well as to reduce the environmental impact.

Gelatines with different characteristics and functional properties could be successfully extracted from enzymatically pre-treated tannery wastes. The optimisation of the extraction temperature allowed tannery wastes to be recycled by obtaining medium grade gelatine suitable for microencapsulation purposes.

KEYWORDS: Biopolymer, enzymes, extraction, microencapsulation, tanning process, valorisation

1 INTRODUCTION

Gelatine is a soluble protein obtained by partial hydrolysis of collagen. The most abundant sources of gelatine are pig skins, bovine hides and pork and cattle bones, and therefore tannery wastes. Currently, within the framework of Directive 2008/98/EC relative to wastes, Member States are required not only to take measures in order to minimise waste production by developing clean technologies, but also to encourage their recovery and valorisation. In this sense, European policy prompted the transformation of tannery solid wastes into valuable co-products which can be recycled or employed in other industries, as for instance for the preparation of organic fertilisers, the production of biomaterials, gelatines or collagens, and the production of biofuel.

Gel-forming properties of gelatine are the basis of classical applications, but its functional properties are also highly appreciated by the pharmaceutical, photographic and cosmetic industries or microencapsulation applications. For instance, gelatine is one of the most used shell-forming materials in the preparation of natural and biodegradable microcapsules [1–3].

Microencapsulation is a coating technology by which active substances are coated in a polymeric shell, leading to core-shell particles called microcapsules. Microencapsulation has a great potential for improving processing ability over a wide range of applications, as well as product performance, particularly in terms of controlled release and enhanced stability against external factors. Indeed, microcapsules, when firmly anchored to a material, can add new smart functionalities without affecting the look and feel of the material. This technology could offer new opportunities for the development of high added value products, promoting brand differentiation and competitiveness [2–6].

One microencapsulation method which uses gelatine as a shell-forming polymer is complex coacervation. This process is based on the phase separation that takes place spontaneously when in an aqueous medium, two or more colloids of opposite charges (a polycation and a polyanion) are mixed in the presence of an active substance dispersion. A protein and a polysaccharide are generally used, such as gelatine and sodium carboxymethylcellulose (CMC) [4, 5, 7].

The optimisation of the extraction conditions of gelatine is necessary in order to obtain specific properties suitable for its use as a shell forming biopolymer for

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microcapsules [1, 7, 8]. For technical applications, such as microencapsulation, gel strength (Bloom strength, related to gelatine's average molecular weight), film-forming or emulsifying properties of gelatine affect to some extent the quality of the microcapsules and, therefore, the microencapsulation process [1].

The process of gelatine extraction from untanned wastes consists of several steps. As a first step, collagen must be acid or alkaline pre-treated in order to produce fibre swelling and collagen solubilisation, thus enabling its extraction. The alkaline pre-treatment is usually preferred when bovine hides are used as a raw material. During this pre-treatment, the non-protein substances as well as the non-collagenous proteins are reliably dissolved out. The alkaline pre-treatment is a long process that takes several months and requires the use of large quantities of water because the alkaline solution has to be replaced repetitively during conditioning and numerous washing processes are necessary to remove alkalis and salts [1]. So this implies the treatment and management of the large amount of wastewater generated. Nowadays, the enzymatic pre-treatment opens up a new alternative to the alkaline pre-treatment to reduce processing time and wastewater.

In this sense, this work aims to recover gelatine from untanned solid wastes in order to use it as a natural microencapsulating agent in the production of active materials with functional properties. Concretely, this paper focuses on the influence of the extraction temperature on gelatine properties and its microencapsulating ability, using an alternative ecoefficient enzymatic process to pre-treat tannery wastes in order to save costs and reduce time, as well as to reduce the environmental impact.

2 EXPERIMENTAL

Limed bovine pelt wastes, provided by a local tannery, were used as a source of gelatine. First of all, bovine hide wastes were washed in tap water with a hide/water ratio of 1:4 (w:v). Then, the washed wastes were pre-treated by adding an alkaline protease supplied by Spain Enzymes under fixed enzymatic conditions. The reaction was carried out using a buffer solution to maintain an alkaline pH. Once the pre-treatment had been completed, the pH was reduced to deactivate the enzyme and avoid further hydrolysis of collagen. Additionally, the influence of the co-use of 0.2 wt% lipase enzyme during the pre-treatment was also tested in order to remove fats from hide wastes and, therefore, its presence in the final gelatine extracted.

After conditioning, the gelatine was extracted under different extraction temperatures (75 °C, 65 °C and 55 °C) and fixed extraction time and pH value (pH = 6.5 for 5.5 h). Additionally, the wastes remaining after the extraction at the lowest temperature were re-extracted by a continuous extraction process at 75 °C in order to increase the gelatine yield. Next, the obtained gelatine solutions were filtered under vacuum and concentrated at constant temperature using a rotavapor device. Finally, the concentrated solution was placed in a PTFE mould and left to dry overnight in a furnace at T = 45 °C to obtain the gelatine films for further characterisation. Table 1 includes the nomenclature of the gelatines at different extraction conditions.

Table 1 Extraction conditions of the gelatines obtained.

Gelatine	Extraction temperature (°C)	Type of extraction
E-75	75	Simple
E-65	65	Simple
E-55	55	Simple
E-75 cont	75	Continued

The yield and properties of the extracted biopolymers were characterised by different experimental techniques.

The extracted gelatines were weighed and their moisture content was determined by thermogravimetric analysis (TGA). Gelatine yield was calculated based on dry waste weight (see Equation 1).

$$Yield\,(\%) = \frac{gelatine\ weight - water\ content}{dry\ waste\ weight} \times 100 \qquad (1)$$

The chemical composition of the gelatines was analysed by infrared spectroscopy (FTIR). Gelatine spectra were obtained in ATR mode using a VARIAN 660-IR spectrometer (Varian Australia PTY LTD, Australia). FTIR spectra were obtained in the spectral range of 4000 to 400 cm^{-1}. The number of scans per experiment was 12 scans at a resolution of 4 cm^{-1}.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used for the evaluation of the molecular weight of the extracted gelatines. The protein pattern of the gelatines was analysed using a 11% polyacrylamide gel. After electrophoresis, proteins in the gel were visualised after staining with 1% (w/v) Coomassie blue in 20% (v/v) acetic acid and 40% (v/v) methanol and subsequent destaining with 20% (v/v) acetic acid, 20% (v/v) methanol and 1% (v/v) glycerol.

One of the most important properties of gelatine is the gel strength or "Bloom" value, which determines the quality of gelatine and its final application. The Bloom value of the extracted gelatines was quantified using a TA-XT Plus texture analyser (Stable Micro

Systems, UK). This value indicates the gel strength of gelatine using a 6.67 wt% solution. This test measures the force required for a standard probe to depress the surface of a gelatine gel to a depth of 4 mm. This test was assessed according to the standard EN ISO 9665 [9].

The microencapsulating ability of the extracted gelatines was evaluated by the complex coacervation method using (R)-(+)-limonene as the core material. The extracted gelatine (polycation) and sodium carboxymethylcelulose (CMC) (polyanion) were used as biodegradable shell-forming polymers. The microencapsulation process was carried out as follows. First, the emulsification of the oil (o/w) was completed in an aqueous gelatine solution at 50 °C, to which a 5 wt% sodium carboxymethylcellulose solution was added. The gelatine/carboxymethylcellulose ratio (G:C) was 10.0 [4]. Then the coacervate formation was induced through the reduction of the pH with a 10 wt% acetic acid solution. Following this, the system temperature was reduced to 5 °C–10 °C. Finally, the hardening step was carried out using a 40 wt% glutaraldehyde solution.

The morphology of the dried microcapsules was analysed by Scanning Electron Microscopy (SEM) using a JEOL JSM-840 microscope (Jeol, Tokyo, Japan). Prior to this, the samples were gold coated to obtain sufficient contrast in the SEM micrographs.

3 RESULTS AND DISCUSSION

The degree of conversion of collagen into gelatine depends on the processing conditions. Table 2 includes the gelatine yield obtained for the different extraction processes. As the extraction temperature increased, the yield increased. Furthermore, a second extraction process at higher temperature (75 °C) of the non-transformed waste obtained at 55 °C, allowed the gelatine yield of this process to be increased.

Figure 1 shows the infrared spectra of the gelatines obtained at different extraction temperatures. The gelatine spectra showed vibration bands at 3400–3100 cm^{-1} for N–H stretching (Amide A and B), 3100–2800 cm^{-1} for alkenyl C–H stretching, 1635 cm^{-1} (Amide I) for C=O stretching, a band at 1550 cm^{-1} (Amide II) for out of phase combination of the N–H in plane bend and the CN stretching vibration, 1480–1300 cm^{-1} for CH_2 bending, 1249 cm^{-1} (Amide III) for in phase combination of the NH bending and CN stretching vibration [10, 11]. Additionally, the extracted gelatines also showed the characteristics bands of fats due to the presence of the carbonyl stretching band of carboxylic groups and also an important increase in C-H stretching bands. The intensity ratio of bands at 1635 cm^{-1} (Amide I of gelatine) to 1745 cm^{-1} (carbonyl of fats) increased as the extraction temperature increased, which could be attributed to a higher gelatine content in the extracted product.

In order to remove fats from hides and to obtain gelatines free from fats, the co-addition of a lipase during the pre-treatment process was also evaluated. Figure 2 shows FTIR spectra of gelatine E55 obtained with and without the use of a 0.2 wt% lipase. The co-use of a lipase during the pre-treatment process removed successfully fats from hide wastes, as it was confirmed by infrared spectra.

Table 2 Gelatine yield data.

Gelatine	Gelatine yield (wt%)
E-75	54.3
E-65	52.0
E-55	32.0
E-75 cont	16.2*

* Apparent yield. Biopolymer obtained after a second extraction at higher temperature during the previous extraction has not been taken into account. Actual yield should be higher.

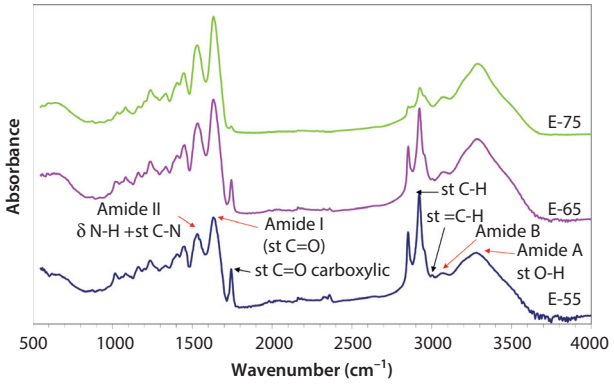

Figure 1 FTIR spectra of gelatines obtained at different extraction temperatures.

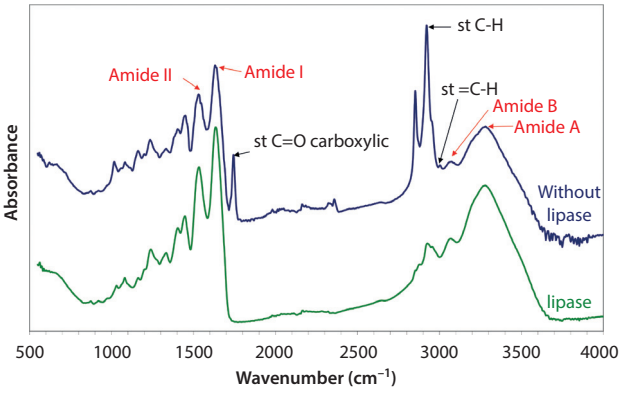

Figure 2 Effect of the co-addition of a lipase during the enzymatic pre-treatment on the chemical composition of a gelatine extracted at 55 °C (E55).

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During gelatine manufacturing, the conversion of collagen into gelatine yields molecules of varying mass, due to the cleavage of inter-chain covalent cross-linkings and the unfavourable breakage of some inter-chain peptide linkages. Gelatine is a mixture of different polypeptide chains including α-chains, β (dimmers of α-chains) and γ (trimmers of α-chains) components with a molar mass that varies between 80–125, 160–250 and 240–375·10^3 g/mol, respectively [8, 11]. Furthermore, gelatine can also contain oliogomers of lower molecular weight as a consequence of the hydrolysis of α-chains [10]. Therefore, gelatines have a polydisperse molecular weight distribution that is related to the collagen structure and its production process.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used for the evaluation of the molecular weight of the extracted gelatines. Figure 3 shows the protein pattern of gelatines extracted from tannery wastes at different temperatures. Extracted gelatines showed a wide molecular weight distribution. Gelatine extracted at the highest temperature (E-75) showed the highest molecular weight components, indicating the presence of α-chains and β-dimmers [10, 12–14]. As the extraction temperature decreased, the presence of molecular weight fractions (α-chains and β-dimmers) decreased in the gelatine extracted from enzymatically pre-treated wastes. This effect is clearly demonstrated in gelatine E-55, extracted at the lowest temperature. E-55 only showed low molecular weight fractions, which were lower than 15 kDa.

This fact can be attributed to type of pre-treatment, which will determine the main solubilisation mechanism of collagen during the extraction stage. During the alkaline pre-treatment of wastes, collagen crosslinkings were cleaved causing the fibres and fibrils of collagen to dissociate into tropocollagen units, that is, only partial hydrolysis took place and collagen proteins remained essentially intact [1]. Next, during the extraction process of alkaline pre-treated wastes, the higher temperatures and longer periods of exposure to heat accelerated the hydrolysis process, producing lower molecular fractions [1, 10, 14]. However, opposite to this mechanism, the enzymatic pre-treatment produced a controlled but higher degree of hydrolysis to allow the subsequent solubilisation of collagen. This alternative mechanism caused the extraction of the easier soluble components of lower molecular weight, taking place first at lower extraction temperatures. Meanwhile, the higher molecular weight fractions were extracted or solubilised at higher extraction temperatures.

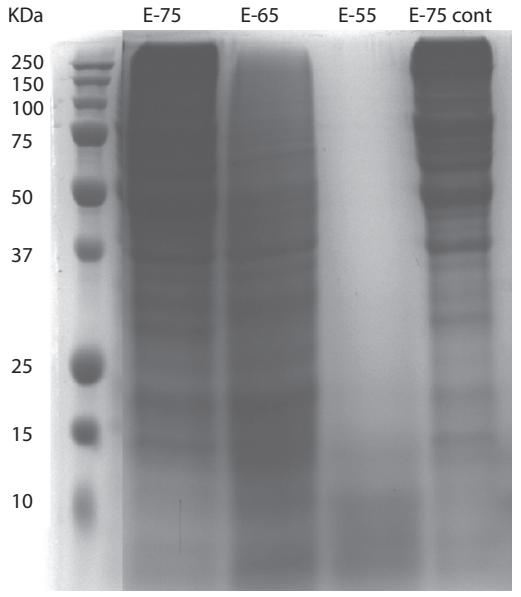

Figure 3 Protein pattern of extracted gelatines, obtained by SDS-PAGE electrophoresis.

Furthermore, the effect of the extraction temperature on the molecular weight is again clearly shown in gelatines extracted at two different temperatures in a continuous process. The gelatine obtained at the lowest temperature (E55) showed the lowest molecular fractions; while the gelatine re-extracted from the remaining non-transformed wastes (E75 cont) clearly showed the presence of higher molecular weight chains. This way, continuous extraction confirmed that lower molecular weight fractions were extracted at lower temperatures and higher molecular weight fractions were extracted at higher ones.

One of the most important properties of gelatine is the gel strength or "Bloom" value, which is associated with the molecular weight and the contents of proline and hydroxyproline in gelatine. Table 3 shows the influence of the extraction temperature on the Bloom value of gelatines. E-75 and E-65 had Bloom values of 135 and 10 g, respectively, while E-55 did not show gelling power. So the results indicated that the Bloom value of gelatine increased as the extraction temperature increased. The difference in gel strength between the samples could be due to the differences in intrinsic characteristics, such as molecular weight distribution and amino acid composition. It is known

Table 3 Bloom value of gelatines obtained at different extraction temperatures.

Gelatine	Bloom value (g)
E-75	135.3 ± 9.3
E-65	10.1 ± 1.1
E-55	No gelling power
E-75 cont	27.1 ± 3.3

that gel strength properties are related to the α-chains and β-chain components of gelatine [1, 10, 12]. The longer chains in E-75 could undergo aggregation to form gel network more effectively than E-65. E-55 gelatine only showed the lowest molecular weight protein fractions and did not contain α-chains and β-chain components, thus avoiding its ability to form the superhelix structure of gelatine gel [1, 10, 12]. According to the results, 75 °C was selected as the most suitable extraction temperature to improve the yield and obtain gelatines with high-medium range Bloom values.

The continued extraction of the non-transformed wastes remaining from the extraction carried out at lower temperature (E75 cont), improved the gelling power of gelatine, which was in concordance with the higher molecular weight obtained. However, the gel strength of E75cont was noticeably lower than that of E75. This fact could be attributed to a higher hydrolysis time of collagen after two consecutive extractions in gelatine.

Furthermore, Bloom strength determines gelatine grade. Gel strength typically ranges between 50–300 [1]. Different gel strength is used for different applications. In technical application, like microencapsulation, usually gelatines with medium to high Bloom values are suitable. Therefore, the gelatine obtained at the highest extraction temperature (E75) showed a medium Bloom value suitable for microencapsulation applications.

The microencapsulating ability of E-75 was evaluated by the complex coacervation process. The SEM images of the microcapsules obtained are shown in Figure 4. Microcapsules were successfully prepared using gelatine extracted from tannery wastes and applying a more ecoefficient enzymatic pre-treatment. A wide particle size distribution of the synthesised microcapsules was observed. The width of the particle size distribution depends on the emulsifiying properties of gelatine, since in this work no other emulsifier was added in the emulsion process during microencapsulation. This way, gelatine acts as a shell-forming material and also as an emulsifier.

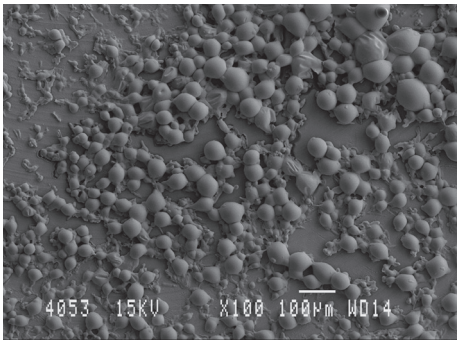

Figure 4 Microcapsules obtained by complex coacervation.

4 CONCLUSIONS

Gelatine from bovine untanned wastes can be successfully extracted as a valuable co-product using an enzymatic pre-treatment, alternative to the conventional alkaline one, to reduce processing time, wastewater and the environmental impact.

The optimisation of the enzymatic pre-treatment of untanned bovine wastes and the extraction temperatures allow medium grade gelatines to be obtained with suitable properties for microencapsulation applications. The extraction temperature determines gelatine yield, gelatine properties and, therefore, its microencapsulating ability.

Currently, successful work on the optimisation of the enzymatic pre-treatment and extraction conditions is being carried out to obtain high grade gelatines from tannery wastes.

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