Tailor-made Biomaterials from Collagenic Wastes

by

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Abstract

The production of biopolymers from leather waste is an important and notable process on the tanning waste management research. The basic aim of the present work is the conversion of non-tanned solid wastes from the tanning industry (splits, defective hides) into high added value biopolymers. Those collagenic biomaterials are at the forefront of potential new applications in tissue engineering within fields such as cosmetics, medicine or veterinary. This treatment will entail a great progress in both environmental and economical terms. The first stage of the project was centered on finding, through a factorial experimental design, the most suitable treatment for the isolation of biopolymers. The effect of variables such as: level of grinding (surface area for chemical interaction); chemical treatment (acid, alkaline and neutral medium); time; temperature and agitation, were studied. Those extracted biomaterials could be formulated according to future application as gel, film, sponge or fibers. All the biopolymers obtained through this process were characterized in order to ascertain the significance of the variables studied. "Tailor-made" biopolymers will be designed, with the desired molecular weight, polymorphic formulation (form), optimized properties and characteristics, that is, perfectly adapted to their future applications.

Resumen

La producción de biopolímeros de los desechos del cuero es un importante y notorio proceso en la investigación del manejo de residuos. El objetivo principal del presente trabajo es la conversión de los desechos sólidos sin curtir de la industria del cuero (carnazas, pieles defectuosas), en biopolímeros de alto valor agregado. Estos biomateriales colagénicos están al frente de nuevas aplicaciones en la ingeniería de tejidos dentro de los campos tales como cosmética, medicina tanto veterinaria como para humanos. Estos procesos generarán grandes logros en términos tanto económicos como ecológicos. La primera etapa del proyecto se centró en hallar por medio de un diseño factorial experimental, el tratamiento más favorable para la obtención de estos biopolímeros. Los efectos de las variables tales: grado de molido (área superficial para reacción química); tratamiento químico (medio ácido, alcalino o neutro); tiempo; temperatura y agitación, fueron estudiados. Tales biomateriales extraídos serían considerados de acuerdo a futuras aplicaciones como gel, película, esponja o fibras. Todos los biomateriales obtenidos a través de este proceso fueron caracterizados para determinar el efecto final de las variables estudiadas. Biopolímeros "hechos a la medida" serán diseñados, con el deseado peso molecular, aspecto polimórfico (forma), óptimas propiedades y características, es decir, perfectamente adaptadas a sus futuros usos.

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INTRODUCTION

The whole world is bending over backwards for the achievement of a more environmentally friendly and sustainable policy. The concept of "Sustainable Development" transmits the idea of the rational use of the resources, the improvement of life quality and the maintenance of the ecosystems without jeopardizing future generations. The improvement of the manufacturing processes, the application of "clean" technologies in the processing, the waste minimization and the finding of new treatments for each type of waste; are essential steps to make compatible industrial development, environmental protection and social welfare. Furthermore, waste treatments could increase their value through the production of high added value products, entailing a great progress in both, environmental and economical terms.

In terms of waste generation, the production of leather gives rise to significant quantities of solid waste product[1, 2] for which tanneries are responsible for the cost of disposal and since most of this waste ends up in landfill it may be considered an environmental problem.[3-5] However, such waste is not without some potential value since it contains collagen that could be recycled and reused. Collagen is a versatile and high-added value protein and the most abundant and ubiquitous in vertebrates.[6, 7] This collagenic nature of the tannery solid waste permits us to think about treatments for obtaining biopolymers of reconstituted collagen fibers, and their use in a wide range of potential applications, including the field of regenerative medicine (tissues and/or organs).[6, 8]

The objective of the present work is the extraction, characterization, optimization and application of new "Tailor-made" smart biopolymers with high added value, finding a new and feasible link between solid tannery waste and the rising market of tissue engineering.

Biopolymers are polymers generated from renewable resources, often biodegradable and from non-toxic production. They are an alternative to the petrol-based polymers and can be produced from biological systems or chemically synthesized from biological raw materials.[9] The main difficulty on the use and application of biopolymers are biocompatibility, mechanical properties and adaptability. Biologically derived materials are advantageous since they contain information that facilitates cell attachment and function, whereas synthetics may not interact with cells in the desired manner.[8, 10]

The importance and special appeal of collagen as a biomaterial is based on the fact that it is a natural material and therefore it is assimilated by the human/animal body as a normal constituent and not as a foreign material, subjected to rejection, with a minimum of inmunogenicity. Collagenic biopolymers present huge possibilities due to the wide range of potential applications. We can talk about "Tailor-made" biopolymers: it is possible to produce easily said biopolymers as gel, film, fibers, tissue and/or sponges, using techniques such as freeze drying/lyophilisation, extrusion, or electro-spinning for nano-fibers formation.

In addition to the technical and scientific benefits obtained from the isolation of biopolymers from solid waste, this research could entail different economical benefits: In the first place, it presents a solution to a problem of dumping/storage of wastes, avoiding taxes for accumulating those wastes. Secondly, whole hides of low quality can be used as raw material; those hides, catalogued as a 4th-5th class, if converted to leather, would be used to produce low quality articles of very low price on the market; however, the biopolymer extracted from this hide would have a higher value. Thirdly, the treatment process is simple and inexpensive. Finally, a wide range of potential applications for the produced bioproducts could be taken into consideration; with specific applications on medicine, veterinary and cosmetics, expanding fields nowadays.

The use of mathematical experimental designs permits to study the degree of significance of the different variables and the corresponding interaction between them in the different processes for obtaining those collagenic biopolymers. This ensures that the experimentation can be rationalized and a mathematical equation controlling the whole process can be defined, this will determine the optimum, being able to achieve a controlled production of "Tailor-made" biopolymers for each specific application.

MATERIALS

Bovine pickled hides supplied by the Leather Technology School of Igualada, were used as a raw material on the biopolymer extraction. Sodium hydroxide (pearl 98-100%) and acetic acid (glacial) were supplied by Carlo Erba and Panreac, respectively. Standard marker for SDS-PAGE (from 6.5 to 205 kDa) was supplied from Bio-Rad. Analytical grade chemicals were used for fiber formation: the phosphate buffer comprised disodium phosphate heptahydrate and monosodium phosphate monohydrate, supplied by Riedel-de Haen and Fluka, respectively. Polyethylene glycol Mw 8000 and sodium chloride were supplied by Sigma and Carlo Erba, respectively.

METHODS

Biopolymer extraction

The basis for the preparation of biopolymer was the degradation of collagen by hydrolysis. The dried hides were cut manually in small pieces and then ground in a grinder

rotor mill (Retsch SR-01) through meshes of different sizes (10 mm, 1 mm and 0.25 mm). Ground bovine hide in a concentration of 50 g hide per liter of hydrolytic solution, were mixed by mechanical stirring (Heidolph stirrer) using different stirring blades. A temperature controlled bath (Lauda E100) with a through-flow cooler attached (Lauda DLK10) was used at a fixed temperature for a determined period of time.

Two studies were designed in order to investigate the effect of the different variables on the biopolymer extraction. The first design investigated the effect of grinding and hydrolysis on the biopolymer extraction process and the second, based on the results of the former one, studied the effect of agitation on the hydrolytic process. The experimental designs were based on Box and Behnken mixed level factorial design, using Statgraphics® software. The values assigned to each variable specified for the design of both studies are shown in Table I.

The analysis of variance (ANOVA) was employed with the aid of Statgraphics® program to obtain the variables with significance above 95%. Graphics of surface and contour were drawn.

TABLE 1
Experimental Design

Study 1: effect of grinding					**Study 2: effect of stirring**		
Sample	**Grinding size (mm)**	**Time (h)**	**Temperature (ºC)**	**Hydrolytic agent**	**Sample**	**Stirrer**	**Speed (rpm)**
1.1	10	6	25	NaOH	**2.1**	Small	525
1.2	0.25	6	25	NaOH	**2.2**	Large	525
1.3	10	48	25	NaOH	**2.3**	Large	50
1.4	0.25	48	25	NaOH	**2.4**	Large	1000
1.5	1	27	5	H_2O	**2.5**	Medium	525
1.6	1	27	45	H_2O	**2.6**	Medium	525
1.7	1	27	5	CH_3COOH	**2.7**	Medium	50
1.8	1	27	45	CH_3COOH	**2.8**	Medium	1000
1.9	10	27	25	H2O	**2.9**	Small	50
1.10	0.25	27	25	H2O	**2.10**	Small	1000
1.11	10	27	25	CH_3COOH	**2.11**	Medium	525
1.12	0.25	27	25	CH_3COOH			
1.13	1	6	5	NaOH			
1.14	1	48	5	NaOH			
1.15	1	6	45	NaOH			
1.16	1	48	45	NaOH			
1.17	10	27	5	NaOH			
1.18	0.25	27	5	NaOH			
1.19	10	27	45	NaOH			
1.20	0.25	27	45	NaOH			
1.21	1	6	25	H_2O			
1.22	1	48	25	H_2O			
1.23	1	6	25	CH_3COOH			
1.24	1	48	25	CH_3COOH			
1.25	1	27	25	NaOH			
1.26	1	27	25	NaOH			
1.27	1	27	25	NaOH			

Fiber formation (extrusion)
The process for fiber formation was based on previous work[8] with slight modifications. A syringe was loaded with biopolymer solution and placed on a syringe pump system supplied by KDScientific (model no: KDS-100-CE). One end of a silicone tube was connected to the syringe and a needle was fitted at the other end and then placed at the bottom of a container. The fibers were extruded into a "Fiber Formation Buffer" (FFB) remaining there for 30 minutes and then transferred into a "Fiber Incubation Buffer" (FIB) for another 10 minutes. Finally, the fibers were air-dried under the tension of their own weight at room temperature. The "Fiber Formation Buffer" comprised 118 mM phosphate buffer and 20% of polyethylene glycol (Mw 8000) at pH 7.55 and 37ºC. The "Fiber Incubation Buffer" comprised 6 mM phosphate buffer and 75 mM sodium chloride at pH 7.10 and 37ºC.

Film formation
Aliquot of the extracted biopolymer (10 ml) was placed in a small Petri dish and allowed to air dry at a constant temperature (20ºC) and relative humidity (60%).

Sponge formation (Lyophilisation)
Samples of the extracted biopolymer were dried by lyophilisation technique, using a freeze drier supplied by Telstar. Samples were frozen in an acetone/dry ice solution prior to the lyophilisation.

Yield
The yield of biopolymer extraction was calculated as the percentage of hide material converted to biopolymer and calculated according to the following formula:

Yield (%) = $100(1-W_{res}/W_{shav})$; where W_{res} is the residual weight of biopolymer after filtration, and W_{shav} is the initial weight of hide.

Swelling[11-13]
The films were weighed and then immersed in a phosphate buffered saline (PBS) solution for different periods of time. Wet samples were blotted with filter paper to remove the surface water not taken into the gel, and re-weighed. The percentage of swelling was calculated as follows:

Swelling (%) = $100(W_{wet} - W_{dried})/W_{dried}$; where W_{wet} is the weight of the film after being immersed in PBS solution for a determined period of time and W_{dried} is the initial weight of the film.

Gel Strength
Gel strength was measured, using 100 ml of gelatin, by Bloom determination which was carried out according to the International Standard (ISO 9665). A Materials Tester designed by Instron, with a 0.5 inch radius cylinder probe (P/0.5R) was used.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Aliquots of 50 mg of gelatin were dissolved in 1 ml of sample buffer. The samples then were denatured at 90ºC for 5 minutes, and loaded in appropriate volumes onto a vertical acrylamide gel (4% (v/v) stacking gel, 7.5% (v/v) resolving gel). A standard marker, from 6.5 to 205 kDa was loaded with the samples. The gels were run at 0.01 mA/gel, stained overnight with Coomassie Brilliant Blue solution, and then destained prior to analysis.

RESULTS AND DISCUSSION

Study 1: Effect of grinding
The biopolymer extraction protocol and the effect of the grinding size were analyzed in the first study. The results of yield and capacity to form fibers and films are shown in Table II.

The yield is an important property in terms of resource management; and film/fiber formation in terms of future applications of the biopolymer. The degree of swelling of the film provides an indication of the matrix network characteristics and the stability of the gelatin films[12, 14].

According with the statistics studies, the variables with significant influence ($p<0.005$; ANOVA) on the yield and the swelling of the biopolymer films, were the grinding size and the hydrolytic agent.

Figures 1a and 1b represent the effect of the significant variables (grinding size and hydrolytic agent) on the yield and swelling of the extracted biopolymer, respectively. The desired response is to maximize the yield and minimize the percentage of swelling. From both Figures 1a and 1b, it can be deduced that the biopolymers with higher yield and minimum percentage of swelling will be obtained with the minimum grinding size (0.25 mm), using as hydrolytic agent acetic acid (CH_3COOH).

The results were analyzed by Multiple Response Optimization, a function that determines the combination of experimental factors that simultaneously optimize several response variables. The goal of the function is to maximize a desirability function. The general approach of the desirability function is to first convert each response into an individual desirability function that varies over the range 0-1 where, if the response is at its goal, then the desirability value is 1, however, if the response is outside an acceptable region, desirability value is 0[14]. The design variables are chosen to maximize the overall desirability from the geometric average of individual desirability's.

Figure 2 confirms the conclusion obtained from figures 1a and 1b; the maximum desirability (desirability = 1) was obtained

with the minimum grinding size (0.25mm) using CH_3COOH as hydrolytic agent.

The significant variables (grinding size and hydrolytic agent) were fixed at their optimum values according to the previous conclusions (Figure 2). Desirability contour plot was drawn (Figure 3) in order to optimize the response of the other two variables in study, temperature and time. The graph shows how these variables, temperature and time, may vary within a wide range nevertheless keeping the responses always inside an acceptable region.

It has been found that the biopolymer with optimum properties (maximum yield and minimum percentage of swelling) can be obtained using hide ground at 0.25 mm, and carrying out the extraction with acetic acid during 24 hours at 10ºC.

Study 2. Effect of agitation (around the optimum)
Based on the result of the previous study, a further investigation around the optimum was carried out in order to ascertain the effect of agitation on the biopolymer extraction procedure. All the samples obtained in this second study presented a 100% yield and were capable to form fibers and films. The results of the agitation on the gel strength and percentage of swelling are showed in Figures 4a and 4b. Gel strength was measured, obtaining information about the level of degradation of the collagen fibers and the formation of a strong network structure.

According with the statistics studies, the variable with significant influence ($p < 0.005$; ANOVA) on the gel strength of the biopolymer was the size of the stirring blade. However, no significant influence on the percentage of swelling was observed for any of the variables studied (Stirrer size and speed of the agitation).

The desirability function analyzed for both responses, gel strength and percentage of swelling, it is represented in Figure 5. The black dot indicates the optimum; the desirability was maximized using a small stirrer blade at low speed on the biopolymer extraction.

It has been found that, in order to obtain a biopolymer with optimum properties (maximum yield and minimum percentage of swelling), the extraction needs to be carried out at low speed (50 rpm), using a small stirrer blade.

Biopolymer extraction (optimum):
A biopolymer was extracted applying the optimum values for the different extraction variables based on the results of the previous studies. The extracted biopolymer presented the following characteristics: yield of 100%, 252 g Bloom, 300% of swelling (after 90 min in solution), and the ability to form films and fibers. The percentage of water absorbed by the films versus time and the molecular weight analysis by SDS-PAGE are represented in Figure 6 and 7, respectively.

TABLE II

Study 1. Results of Yield, and fiber and film formation

Sample	Yield (%)	Fiber formation	Film formation
1.1	2.2	No	No
1.2	100.0	No	Yes
1.3	2.5	No	No
1.4	100.0	No	Yes
1.5	8.7	No	No
1.6	23.2	No	Yes
1.7	36.3	No	Yes
1.8	94.8	Yes	Yes
1.9	4.7	No	No
1.10	100.0	No	Yes
1.11	3.0	No	No
1.12	100.0	Yes	Yes
1.13	96.3	No	Yes
1.14	47.6	Yes	Yes
1.15	94.3	No	No
1.16	100	No	Yes
1.17	0.5	No	No
1.18	100.0	No	Yes
1.19	100.0	No	Yes
1.20	100.0	No	Yes
1.21	7.1	No	No
1.22	12.6	No	No
1.23	100.0	Yes	Yes
1.24	100.0	Yes	Yes
1.25	100.0	No	Yes
1.26	82.2	Yes	Yes
1.27	81.8	Yes	Yes

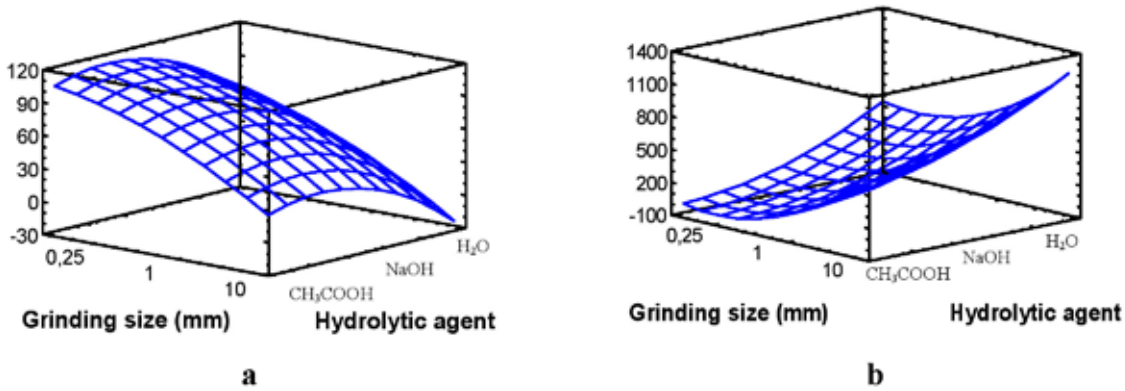

Figure 1: Surface plot of the effect of the grinding size and hydrolytic agent on the yield (a) and the percentage of swelling (b) of extracted biopolymer.

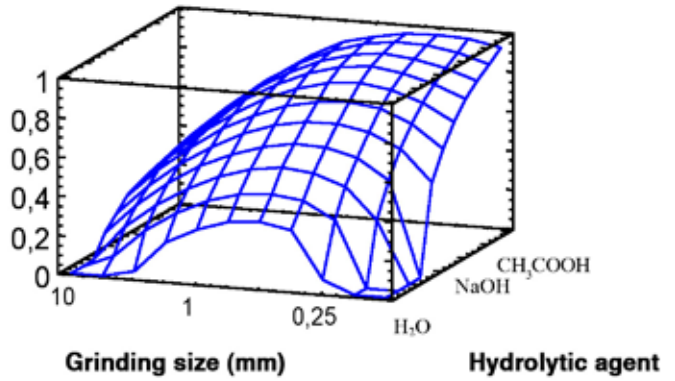

Figure 2: Surface plot of the effect of grinding size and hydrolytic agent on the desirability of biopolymer extraction procedure (optimum at desirability = 1).

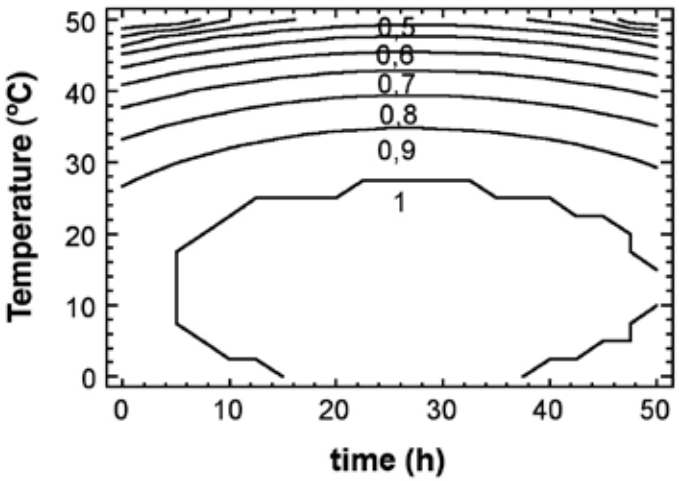

Figure 3: Desirability contour plot of the effect of time and temperature on the biopolymer extraction procedure.
(Grinding size = 0.25mm; Hydrolytic agent: CH_3COOH)

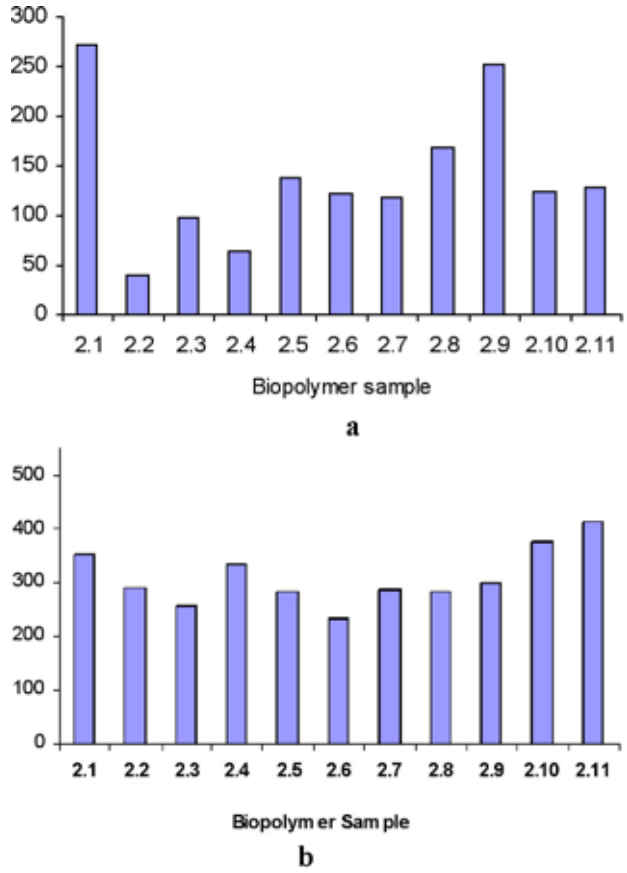

Figure 4: Gel strength (a) and percentage of swelling (b) values for biopolymers extracted according 2nd study (effect of agitation).

Collagen isolated from various tissues has a molecular weight of about 300kDa[6]. For collagen derivatives, the molecular weight usually ranges within limits of 15-50kDa for hydrolysates[16] and 50-200 kDa for gelatin[17]. Collagenic biopolymers usually show a wide molecular weight distribution on the low molecular weight area due to the process of extraction, which leads into a material consisting of different molecular weight polypeptides18; however, the results of the electrophoresis of the biopolymer extracted according to our methodology exhibit (Figure 7) two distinct bands in the high

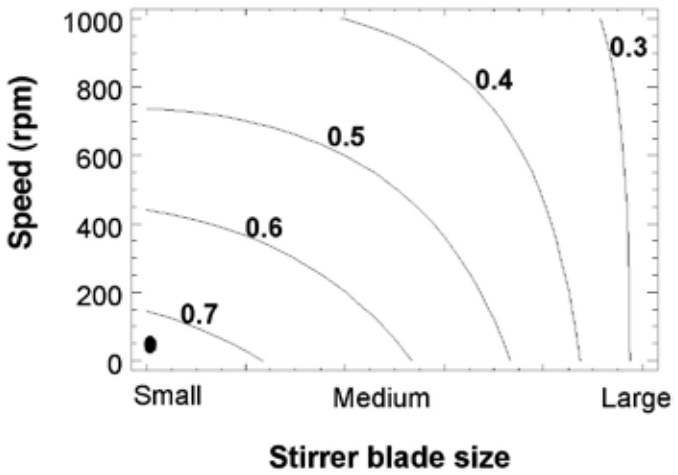

Figure 5: Desirability contour plot of the effect of stirrer blade size and stirring speed on the biopolymer extraction procedure. ● indicates the optimum.

molecular weight area (150-250 kDa) showing low degradation of the collagen triple helix during the extraction process.

Biopolymer polymorphic formulations:
The extracted biopolymer can be easily formulated as gels, film/tissue, porous scaffolds/sponges and fibers (Figure 8).

After the extraction, the biopolymer was obtained as a gel (Figure 8a). Collagen gels are very attractive for tissue engineering applications because they can retain cells and carry bioactive molecules such as growth factors[6]. Films (Figure 8b) can be obtained just air-drying the gel (25ºC and 60% RH). Collagen sponges (Figure 8c) are generally formed by freeze-drying an aqueous collagen solution. The freezing temperature and freezing rate will have some effect on the porous structure of the resulting sponge[6].

The traditional process for formation of collagen fibers (Figures 8d and 8e) involved the extrusion of collagen dispersions into a fiber formation buffer and subsequent solvent dehydration, air-drying and crosslinking. In addition to the traditional extrusion procedure for obtaining collagen fibers, for future studies electro-spinning shows to be a promising technique to manufacture in vitro fibrous scaffold for tissue engineering applications. The incorporation of crosslinking can be used to further tailor and control the material properties of the fibers, sponge/scaffold to specific applications.

CONCLUSIONS

The possibility of obtaining new high-added value biomaterials from solid leather waste, more specifically from low quality bovine hides, has been demonstrated. A complete methodology for the extraction of new biopolymers from tannery solid

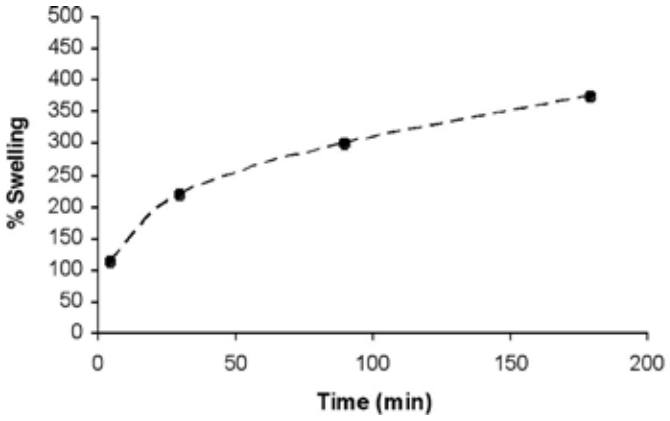

Figure 6: Percentage of swelling vs. time for the extracted biopolymer.

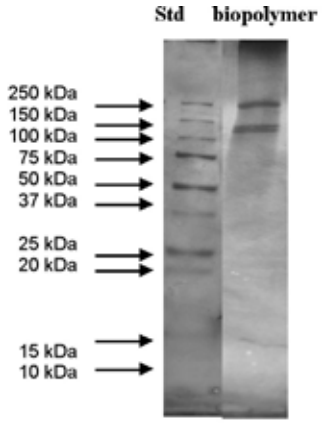

Figure 7: Molecular weight analysis of the extracted biopolymer using SDS-PAGE.

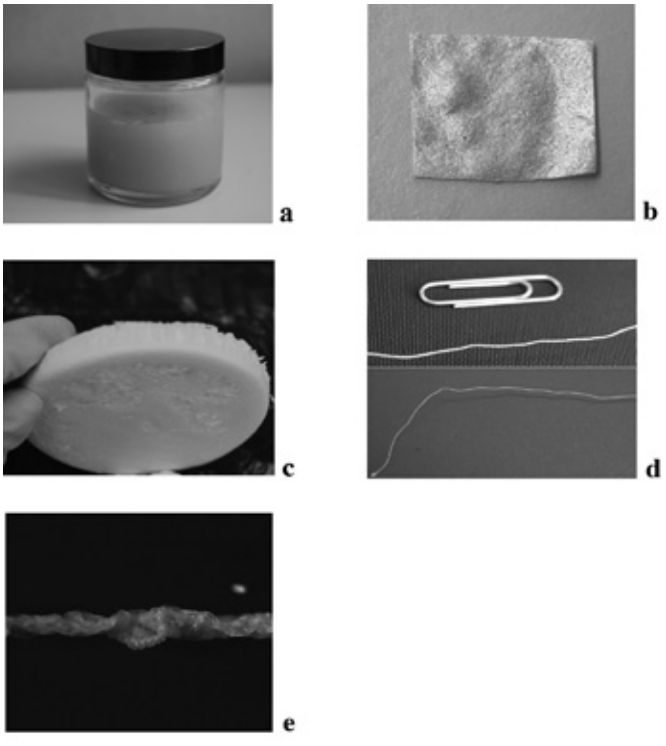

Figure 8: Different polymorphic formulation for extracted collagenic biopolymer: (a) gel; (b) film; (c) sponge; (d) extruded fiber; (e) extruded fiber by optical microscopy (diameter 300μm)

waste has been developed. It has been demonstrated that those biopolymers can be produced in form of gels, films, sponges and/or fibers with the desired characteristics of yield, swelling and gel strength, showing that reconstituted collagen is a competitive biomaterial. Therefore, opening up a new potential future market in the fields of cosmetics, medicine or veterinary will entail both important economical and environmental benefits.

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REFERENCES

1. Alexander, K. T. W. *The conversion of solid tannery waste into saleable by-products; Workshop on pollution abatement and waste management in the tanning industry for countries of the Danube river basin*. 1995
2. Cabeza, L., Taylor, M. M., DiMaio, G. L., Brown, E., Marmer, W. N., Carrió, R., Celma, J. and Cot, J., *Waste Manage*. **18**, 211-218, 1998
3. Hughes, J. C., *Soil Use Manage*. **4**, 107-111, 1988
4. Basegio, T., Haas, C., Pokorny, A., Bernardes, A.M. and Bergmann, C.P., *J Hazard Mater*. **137**, 1156-1164, 2006
5. Babu, N. K. C., Asma, K., Raghupathi, A., Venba, R., Ramesh, R. and Sadulla, S., *J Clean Prod*. **13**, 1189-1195, 2005
6. Reis, R. L., Neves, N. M., Mano, J. F., Gomes, M. E., A.P.Marques and Azevedo, H. S. Woodhead Publishing, CRC Press. 2008
7. Gelse, K., Pöschl, E. and Aigner, T., *Adv Drug Deliver Rev*. **55**, 1531-1546, 2003
8. Zeugolis, D., Paul, R. G. and Atenburrow, G., *J Biomed Res A,* **86A**, 892-904, 2008
9. Langer, R. and Vacanti, J. P., *Science* **260**, 920-926, 1993
10. Bigi, A., Cojazzi, G., Panzavolta, S., Roveri, N. and Rubini, K., *Biomaterial* **23**, 4827-4832, 2002
11. Bigi, A., Panzavolta, S. and Rubini, K., *Biomaterial*, **25**, 5675-5680, 2004
12. Zeugolis, D. I., Paul, R. G. and Attenburrow, G., *J Biomed Mat Res A,* **89**, 865-908, 2009
13. Bigi, A., Bracci, B., Cojazzi, G., Panzavolta, S. and Roveri, N., *Biomaterials* **22**, 763-768, 2001
14. Vílchez, S., Manich, A. M., Jovancic, P. and Erra, P., *Carbohyd Polym*. **71**, 515-523, 2008
15. Langmaier, F., Mokrejs, P., Karnas, R., Mládek, M. and Kolomazník, K., *J Soc Leath Tech Ch*. **90**, 29-34, 2006
16. Brown, E., Thompson, C. and Taylor, M. M., *JALCA* **89**, 215-220, 1994
17. Zhongkai, A., Guoying, L. and Bi, S., *J Soc Leath Tech Ch*. **90**, 23-28, 2006