Electronic Supporting Information

Tailor-made biopolymers from leather waste valorisation

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MATERIALS AND METHODS:

Two types of bovine hides were studied as raw material: splits of pickled hides and crust, which were supplied by the Leather Technology School of Igualada (Spain). Sodium hydroxide (pearl 98-100%) was supplied by Carlo Erba and acetic acid (99.5% PS) and ammonia (25% PA) were supplied by Panreac. Standard marker for SDS-PAGE (from 6.5 to 205 kDa) was supplied from Bio-Rad. Analytical grade chemicals were used for fibre 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. Ethylene Glycol Diglicydil Ether (EGDE), 5-ethyl-1-aza-3,7-dioxabicyclo[3,3,0] octane (oxazolidine II), and phosphate buffered saline (PBS) solution were of analytical grade and supplied by Sigma-Aldrich.

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. Three 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 last study was focused around the optimum of the previous studies, as well as the effect of the use of an acidic, alkaline or neutral hydrolytic agent; this study was carried out using two different raw materials (splits and crust hides). 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 the studies are shown in Table S1.

Percentage of centrifuged (Yield): all the samples were centrifuged at 5000rpm for 15 minutes. The supernatant was poured out and percentage of centrifuged biopolymer was

calculated as follows: Centrifuged (%) = $100(W_{centrifuged\ residue})/W_{initial\ sample}$; where $W_{centrifuged\ residue}$ is the weight of the sample after pouring out the supernatant and $W_{centrifuged\ residue}$ is the initial weight of the sample prior centrifugation.

Swelling: 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.

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%).

Fibre formation (extrusion): The process for fibre 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 fibres were extruded into a "Fibre Formation Buffer" (FFB) remaining there for 30 minutes and then transferred into a "Fibre Incubation Buffer" (FIB) for another 10 minutes. Finally, the fibres were air-dried under the tension of their own weight at room temperature. The "Fibre Formation Buffer" comprised 118 mM phosphate buffer and 20% of polyethylene glycol (Mw 8000) at pH

7.55 and 37 ºC. The "Fibre Incubation Buffer" comprised 6 mM phosphate buffer and 75 mM sodium chloride at pH 7.10 and 37 ºC.

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. This test determines the force necessary for a probe to deflect the surface of the gelatin by 4 mm without breaking the gel. Gel strength results were expressed as grams Bloom.

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.

Crosslinking: All the crosslinking reactions were carried out at an equimolar concentration of 0.07M (equivalent to the GTA concentration 0.625% (v/v)15-17 found in literature). The different crosslinking solutions were prepared as follows: Oxazolidine II was prepared in 0.01M PBS (pH 7.4)18, 19.The EGDE solution was prepared by diluting the crosslinker in 0.025M di-sodium tetraborate solution (pH 9.0)20. Samples were crosslinked by immersing the already prepared uncrosslinked extracted gelatin films in the

different crosslinking reagent solutions for 24 hours at room temperature. All the films were repeatedly washed with deionised water once the crosslinking reaction had been completed, air dried at room temperature and then stored at 20ºC and 65% RH until required.

Ultrafiltration: The ultrafiltration unit operation (Figures S1a and b) consists of a pump to generate flow of the feed stream through the membrane-ultrafiltration module. During each pass of fluid over the surface of the membrane, the applied pressure forces a portion of the fluid through the membrane and into the permeate stream. A Prep/Scale spiral filter cartridge (Figure S1c) of 100kDa pore size supplied by Millipore was used. The cartridge was cleaned with a solution of sodium hydroxide 0.1M at 45ºC for at least 2 hours and then rinsed with distilled water. The biopolymer solution was diluted in order to decrease its viscosity and allow the pass through the membrane and then poured into the "feed container". The pump (Procon® pump supplied by Millipore) was set up at low speed and the pressure was adjusted using the retentate valve at 10-20 psi. After the use, the membrane was cleaned with distilled water and sodium hydroxide (0.1M) solution at 45ºC; the ultrafiltration cartridge was filled with storage solution (sodium hydroxide 0.1 M) until the next run to prevent organism growth and drying of the membranes.

Multiple Response Optimization (Surface response methodology) is a function that determines the combination of experimental factors that simultaneously optimize several response variables; the goal of the function being the maximisation of 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.[21] The design variables are chosen to maximise the overall desirability from the geometric average of individual desirabilities. The desired responses are to maximise the percentage of centrifuged (yield) and minimise the percentage of swelling.

Table S1. Experimental designs applied for the different variables in study

Study 1: effect of stirring					Study2: agitation			Study 3: around optimum			
	Grinding size (mm)	Time (h)	Temp (ºC)	Agent		Stirrer	Speed (rpm)		Agent	Time (h)	Temp (ºC)
1.1	10	6	25	NaOH	**2.1**	Small	525	**3.1**	H_2O	16	5
1.2	0.25	6	25	NaOH	**2.2**	Large	525	**3.2**	CH_3COOH	8	25
1.3	10	48	25	NaOH	**2.3**	Large	50	**3.3**	H_2O	24	15
1.4	0.25	48	25	NaOH	**2.4**	Large	1000	**3.4**	H_2O	16	25
1.5	1	27	5	H_2O	**2.5**	Medium	525	**3.5**	CH_3COOH	24	25
1.6	1	27	45	H_2O	**2.6**	Medium	525	**3.6**	CH_3COOH	8	5
1.7	1	27	5	CH_3COOH	**2.7**	Medium	50	**3.7**	NaOH	24	15
1.8	1	27	45	CH_3COOH	**2.8**	Medium	1000	**3.8**	NaOH	8	15
1.9	10	27	25	H_2O	**2.9**	Small	50	**3.9**	CH_3COOH	24	5
1.10	0.25	27	25	H_2O	**2.10**	Small	1000	**3.10**	NaOH	16	25
1.11	10	27	25	CH_3COOH	**2.11**	Medium	525	**3.11**	NaOH	16	5
1.12	0.25	27	25	CH_3COOH				**3.12**	H_2O	8	15
1.13	1	6	5	NaOH				**3.13**	CH_3COOH	16	15
1.14	1	48	5	NaOH				**3.14**	CH_3COOH	16	15
1.15	1	6	45	NaOH				**3.15**	CH_3COOH	16	15
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							

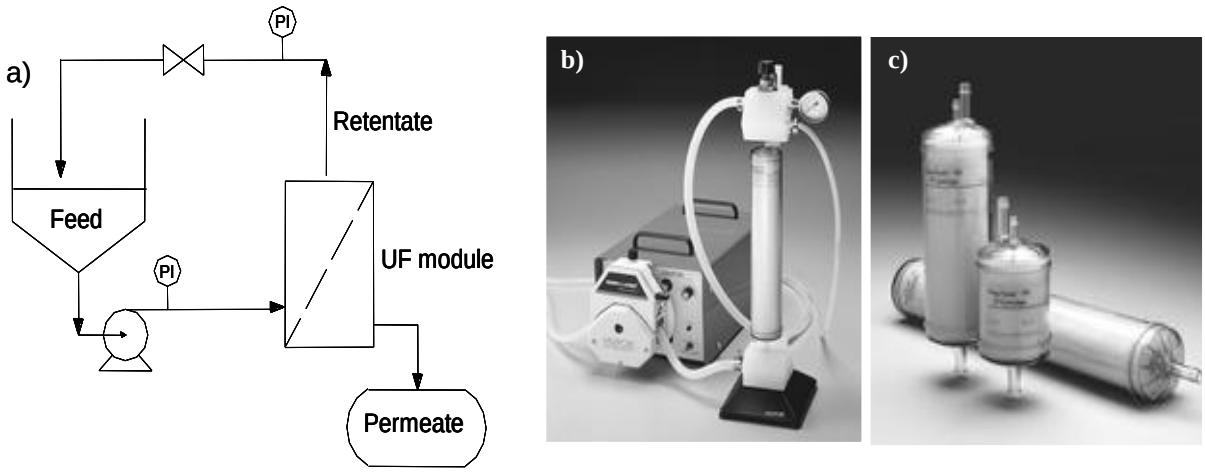

Figure S1. a) Scheme of the ultrafiltration unit; b) Picture of the actual ultrafication unit; c) ultrafiltration prep-scale cartridges.