

# FACTORIAL EXPERIMENTAL DESIGN APPLIED TO THE EXTRACTION OF BIOPOLYMERS FROM NON-TANNED WASTE

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**Abstract:** The basic line of our research is the treatment of industrial waste, with the aim of solving environmental problems. Furthermore, the possibility of obtaining new high added value materials from those said industrial wastes will entail a great progress in both environmental and economical terms.

The aim of the present work is the extraction of a collagen-based biopolymer from collagenic wastes with a minimum hydrolytic effect on the triple helix of the collagen molecule. Defective hides, due to a bad conservation or to the genetic nature of the animal, were used as a raw material in this work.

A Box-Behnken factorial experimental design was used to estimate all the main, quadratic and two-way interaction effects between all the variables in study. The first significant variable in study was the level of grinding; the hides need to be ground in order to increase the surface area for chemical interaction. Treatments with hide fibres passed through meshes of different sizes (10 mm, 1 mm and 0.25 mm) were carried out. The chemical treatment was performed in acid, alkaline and neutral medium, using acetic acid, sodium hydroxide as hydrolytic reagents. Time and temperature were also studied as variables in this design.

All the biopolymers obtained through this process were characterised determining their chemical and mechanical properties, in order to ascertain the significance of the variables studied. From those results, a biopolymer with optimum properties (and higher value than the manufactured product) could be obtained, for future potential applications in fields such as cosmetics, medicine or veterinary.

**Key words:** biopolymer, waste, collagen.

## 1. Introduction

The whole world is bending over backwards the achievement of a more environmentally friendly and sustainable policy. In terms of waste management, this improvement can be achieved by the development of better manufacturing processes, the application of “clean” technologies in the processing, the minimisation of the waste and/or the use of new treatments for each type of waste.

If those treatments, in addition to reduce the volume of industrial wastes, the increase of their value through the production of high-added value products will entail a great progress in both, environmental and economical terms. This is the main aim of this Project, to find a suitable treatment for the conversion of some industrial wastes into new and smart high-added value biopolymers, for its application in fields such as cosmetics, medicine/veterinary or technical applications (bioplastics).

Nowadays, tanning wastes are important and problematic due to the large volume generated and the nature of the wastes (variety of chemicals, chrome solid waste, liquid waste...). It should be reminded that one tonne of wet salted hide yields only 300kg of leather, but over 700kg of solid waste<sup>1</sup>.

The collagenic nature of those solid wastes from the tanning industry is the base of their wide range of potential applications. The high content on collagen<sup>2</sup>, a very special high-added value protein and one of the most abundant in animals, permits to think about treatments for obtaining biopolymers and their application in fields such as cosmetics, medicine, veterinary, etc.

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Biopolymers are polymers generated from renewable resources, often biodegradable and from non-toxic production. They can be produced from biological systems or chemically synthesised from biological raw materials<sup>3</sup>. They are an alternative to the petrol-based polymers. The main problems of biopolymers are: bio-compatibility, mechanical properties and adaptability.

Collagenic biopolymers present huge possibilities, both due to the great variety of composites, and to the possibility of manufacture and application in different ways, with well determined characteristics: we can talk about “Taylor-made” biopolymers. It is possible to produce easily said biopolymers in gel, film, fibres, tissue and/or sponges, using techniques such as freeze drying/lyophilisation, extrusion or electro-spinning for nano-fibres formation.

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 rationalised and a mathematical equation controlling the whole process can be defined, this will determine the optimum in each case, being able to achieve a controlled production of “Taylor-made” biopolymers for each specific application.

## 2. Experimental

### 2.1. Materials

Bovine pickled hides supplied by the Leather Technology School of Igualada, were used as a raw material for the biopolymers 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.

### 2.2. Biopolymer extraction

The basis for the preparation of biopolymer-gelatin was the degradation of collagen by hydrolysis. Grinded bovine hide in a concentration of 50 g hide per liter of hydrolytic solution, were mixed by mechanical stirring (Heidolph stirrer), in a temperature controlled bath (LAUDA E100) with a through-flow cooler attached (LAUDA DLK10), at a fixed temperature for a determined period of time.

Two studies were designed in order to study the effect of the different variables on the biopolymer extraction. The second study was based on the results of the former one. An experimental design based on Box and Behnken mixed level factorial design using Statgraphics® software, was applied. The values assigned to each variable specified for the design of both studies are shown in Tables 1 and 2.

**Tab. 1 Study 1\_effect of grinding: Experimental levels applied for the different variables.**

Variable	Low level	Medium level	High level
Grinding size (mm)	0.25	1	10
Time (h)	6	27	48
Temperature (°C)	5	25	45
Hydrolytic agent	H <sub>2</sub> O	NaOH	CH <sub>3</sub> COOH

**Tab. 2 Study 2\_effect of stirring. Experimental levels applied for the different variables.**

Variable	Low level	Medium level	High level
Stirrer blade size	small	medium	large
Speed (rpm)	50	525	1000

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.

## **2.4 Characterisation**

### **2.4.1. Yield**

The yield of biopolymer was calculated as the percentage of leather material converted to biopolymer and calculated according to the following formula:  $\text{Yield (\%)} = 100(1 - W_{\text{res}}/W_{\text{shav}})$ . Where  $W_{\text{res}}$  is the residual weight of biopolymer after centrifugation/filtration, and  $W_{\text{shav}}$  is the initial weight of hide.

### **2.4.2. 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 tissue to remove excess liquid and re-weighed. The amount of absorbed water was calculated as follows:

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

### **2.4.3. SDS-PAGE**

Aliquots of 50 mg of gelatin were dissolved in 1 ml of sample buffer. The samples then were denatured at 90°C 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. Gels were stained overnight with Coomassie Brilliant Blue solution, and then destained with methanol (10% (v/v)) and acid acetic (7% (v/v)) solution prior to analysis.

### **2.4.4. Gel Strength**

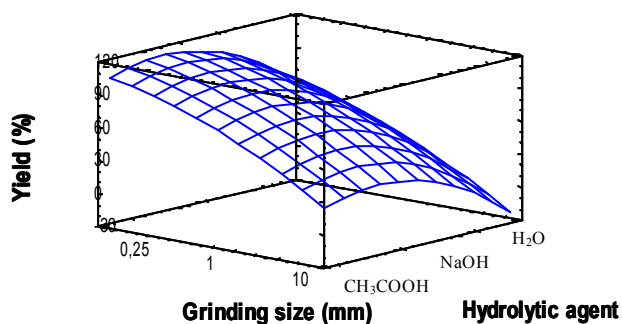
Gel strength was measured, using 100 ml of gelatin, by Bloom determination which was carried out according to the International Standard (ISO 9665). A Materials Tester designed by Instron, with a 0.5 inch radius cylinder probe (P/0.5R) was used. This test determines the force necessary for a probe to deflect the surface of the gelatin by 4 mm without breaking the gel. Gel strength results were expressed as grams Bloom.

## **3. Results and discussion**

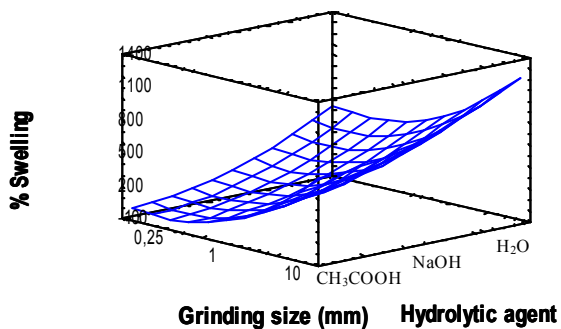
### **• 1<sup>st</sup> study: effect of grinding:**

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

Figures 1 and 2 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 to maximise the yield and minimise the percentage of swelling, from both Figures 1 and 2, 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 ( $\text{CH}_3\text{COOH}$ ).

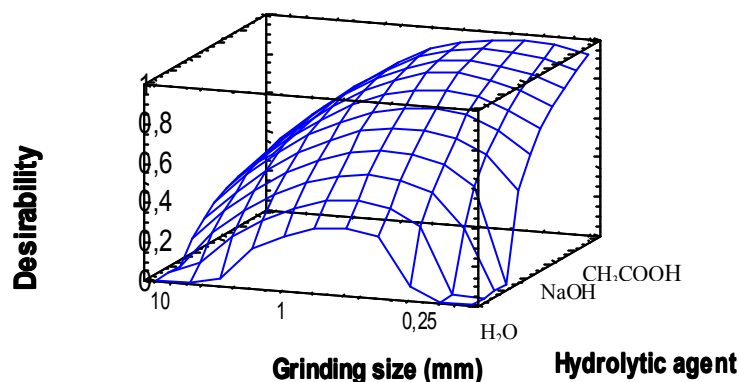


**Fig. 1** Surface plot of the effect of the grinding size and hydrolytic agent on the yield of extracted biopolymer.



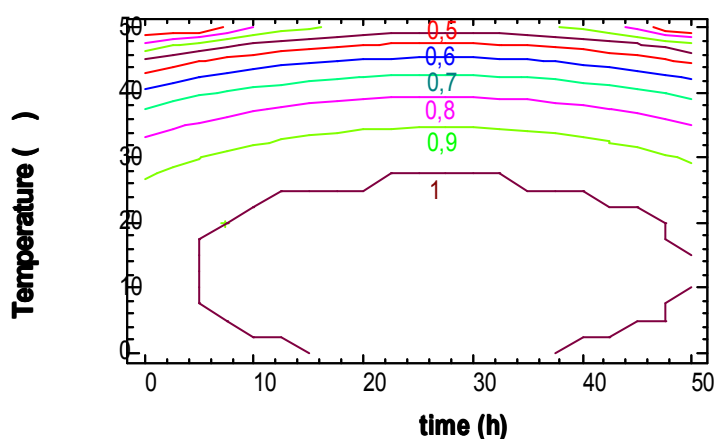
**Fig. 2** Surface plot of the effect of the grinding size and hydrolytic agent on the percentage of swelling of extracted biopolymer.

The results were analysed 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<sup>4</sup>. The design variables are chosen to maximise the overall desirability from the geometric average of individual desirabilities. Figure 3 confirms the conclusion obtained from figures 1 and 2; the maximum desirability (desirability = 1) was obtained with the minimum grinding size (0.25mm) using CH<sub>3</sub>COOH as hydrolytic agent.



**Fig. 3** Surface plot of the effect of grinding size and hydrolytic agent on the desirability of biopolymer extraction procedure (optimum at desirability = 1).

The significant variables (grinding size and hydrolytic agent) were fixed at their optimum values according to the previous conclusions (Figure 3). Desirability contour plot was drawn (Figure 4) in order to optimise 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.



**Fig. 4 Desirability contour plot of the effect of time and temperature on the biopolymer extraction procedure.**  
(Grinding size = 0.25mm; Hydrolytic agent: CH<sub>3</sub>COOH)

Table 3 summarizes the optimum values for each variable to optimise each response as well as the overall optimisation. It has been found that the biopolymer with optimum properties can be obtained using hide grinded at 0.25 mm, and carrying out the extraction with acetic acid during 24 hours at 10°C.

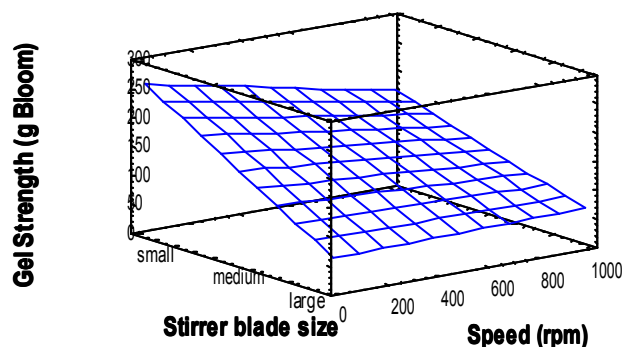
**Tab. 3 Optimum values of variables for biopolymer extraction procedure (study1\_effect of grinding).**

Variable	Optimum (maximise yield)	Optimum (minimise % swelling)	Optimum
Grinding size (mm)	0.25	0.25	0.25
Time (h)	24	26	24
Temperature (°C)	5	14	10
Hydrolytic agent	CH <sub>3</sub> COOH	CH <sub>3</sub> COOH	CH <sub>3</sub> COOH

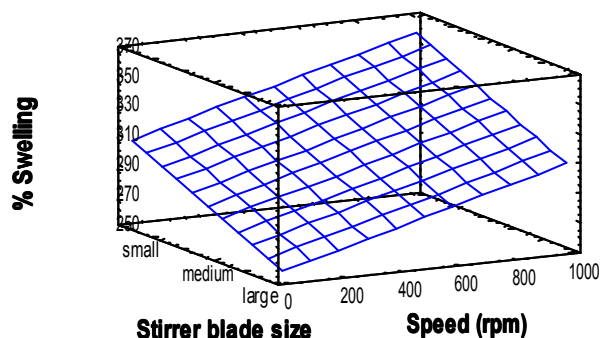
- **2<sup>nd</sup> study: effect of agitation (around the optimum)**

All the samples obtained in this second study presented a 100% yield.

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).

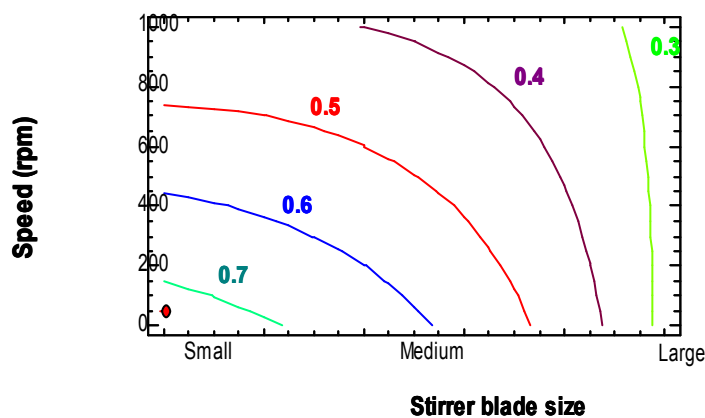


**Fig. 5 Surface plot of the effect of stirrer blade size and stirring speed on the gel strength of extracted biopolymer.**



**Fig. 6 Surface plot of the effect of stirrer blade size and stirring speed on the percentage of swelling of extracted biopolymer.**

The desirability function analysed for both responses, gel strength and percentage of swelling, it is represented in Figure 7. The red dot indicates the optimum, the desirability was maximised using a small stirrer blade at low speed on the biopolymer extraction.



**Fig. 7 Desirability contour plot of the effect of stirrer blade size and stirring speed on the biopolymer extraction procedure. ● indicates the optimum.**

Table 4 summarizes the optimum values for each variable to optimise each response as well as the overall optimisation. It has been found that, in order to obtain a biopolymer with optimum properties, the extraction needs to be carried out at low speed, using a small stirrer blade.

**Tab. 4 Optimum values of variables for biopolymer extraction procedure (study2\_effect of agitation).**

Factor	Optimum (maximise gel strength)	Optimum (minimise % swelling)	Optimum
<b>Stirrer blade size</b>	Small	Large	Small
<b>Speed (rpm)</b>	50	50	50

#### 4. 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 biopolymer with optimum characteristics of yield, swelling, gel strength has been extracted. The properties of the extracted biopolymer make it suitable for the production of films and fibres. 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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