Rational design and characterization of bioplastics from Hermetia illucens prepupae proteins

Original
Rational design and characterization of bioplastics from Hermetia illucens prepupae proteins / Barbi, S., Messori, M., Manfredini, T., Pini, M., Montorsi, M.. - In: BIOPOLYMERS. - ISSN 0006-3525. - (2019), pp. 1-12. [10.1002/bip.23250]

Publisher:
John Wiley & Sons

Published
DOI:10.1002/bip.23250

Rational design and characterization of bioplastics from Hermetia illucens prepupae proteins

Journal:	*Biopolymers*
Manuscript ID	Draft
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Barbi, Silvia; Universita degli Studi di Modena e Reggio Emilia Dipartimento di Scienze e Metodi dell Ingegneria, Dipartimento di Scienze e Metodi dell'Ingegneria Messori, Massimo; Universita degli Studi di Modena e Reggio Emilia Dipartimento di Ingegneria Enzo Ferrari, Department of Engineering Enzo Ferrari Manfredini, Tiziano; Universita degli Studi di Modena e Reggio Emilia Dipartimento di Ingegneria Enzo Ferrari, Department of Engineering Enzo Ferrari Pini, Martina; Universita degli Studi di Modena e Reggio Emilia - Sede di Reggio Emilia, Department of Science and Methods for Engineering Montorsi, Monia; Universita degli Studi di Modena e Reggio Emilia - Sede di Reggio Emilia, Department of Science and Methods for Engineering
Keywords:	biopolymers, recycling, proteins, waste, mechanical properties

Response to Reviewers

Response to Comments from the Reviewer #1

We appreciate that the reviewer's comments. Considering the generic comment, the authors desire to underline that the samples obtained through the DoE are too different to be measured with the same experimental method of measurement, therefore no significant results from the statistical point of view can be drawn. The followings are our point-by-point responses (in italics the manuscript text, highlighted in yellow the new parts):

Comment #1:

If the functional role of GL and CA overlaps, the authors should prepare samples without GL also.

Response

We agreed with the Reviewer and we prepared samples without GL in a preliminary part of the work before the experimental plan. In paragraph 2.1 Design of Experiments more information about these preliminary tests and the ranges chosen to prepare samples for the DoE analysis were provided.

"The ranges as expressed in Table 1 were chosen in consideration of preliminary tests done on protein/GL and protein/CA samples employing different amounts of DI. In the case of protein/GL mixtures a very positive result was obtained in terms of ability to constitute a free-standing and flexible film, for samples containing at least 50 wt% of glycerol. On the opposite, protein/CA samples demonstrated good capacity to form a cohesive material but with loss of flexibility increasing the amount of CA. Therefore, a structural plan of experiments considering all the three factors was needed."

Comment #2:

The authors suggested application in agriculture but, as the authors stated, it does not have enough mechanical properties. At least, the degradation profile against time should be performed.

Response

This works represents one of the firsts studies about the possible employment of protein from insects for mulch film production and nevertheless the mechanical properties are not equivalent to the ones of industrialized products, the perspective of their employment in agriculture are interesting for purposes where a restrained strain at break is needed. As suggested by the reviewer the degradation profile against time is one of the main properties to be measured in order to estimate the lifetime of a product for agriculture purposes. Nevertheless, only very recently the European Union provided a specific regulation about the test methods and requirements for biodegradable mulch films for use in agriculture and horticulture with EN 17033 (January 2018) . In particular, in the Annex G of EN 17033, methods for the evaluation of the degradation rate and lifetime due to different factor such as artificial weathering and soil conditions are defined. Therefore, in the present paper a further characterization about the exposure to artificial weathering during time has been performed in accordance with EN 17033:2018 and EN ISO 4892-2:2013 Method A Cycle 1. The method employed was described at the end of paragraph 2.4 Measurement and the results presented in paragraph 3.2. Characterization of Bioplastic Films Based on Different Amount of Proteins.

In paragraph 2.4 Measurement the following statement was added:

"The degradation profile against time was evaluated on selected samples according with EN 17033:2018 and EN ISO 4892-2:2013 Method A cycle 1 for the measurement of the degradation due to artificial weathering. Rectangular specimens (50x15mm) were exposed in a closed chamber at irradiance of 0,51 W/(m^2 x nm), with fixed temperature equal to 38°C and relative humidity equal to 65% continuously for 500 hours. Deionized water was sprayed during the exposure with cycle of 18 minutes over 2 hours. Mechanical properties were measured after the exposure."

In paragraph 3.2. Characterization of Bioplastic Films Based on Different Amount of Proteins a new figure representing the results was added (Figure 8) together with the discussion as follows:

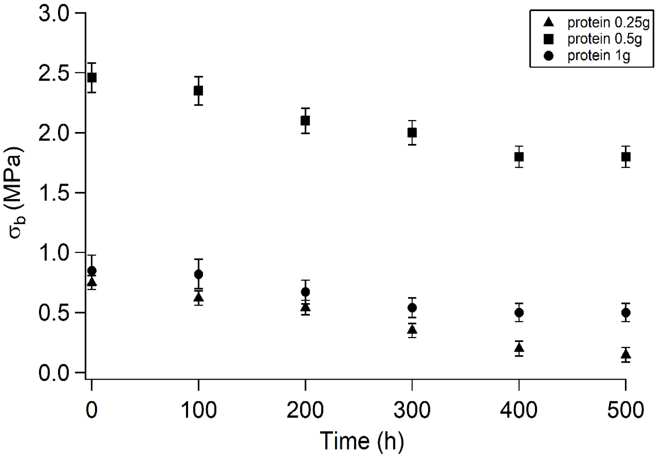


Figure 8 – Profile degradation of tensile stress at break Vs Time

"*The degradation profile against time of three selected samples have been measured as shown in Figure 8. Sample containing 0.5 g of protein has been chosen due the highest tensile stress at break measured, instead the other two samples have been tested as comparison of two opposite situation, both representative of a decreasing tensile stress at break, as shown in Figure 7 and previously discussed. As expected, a decreasing of the tensile stress at break with the increasing of the time under exposure to accelerated weathering has been measured for all the samples investigated, with very similar rate of degradation. Nevertheless, a decreasing rate of degradation can be observed moving from the sample containing 0.25g of protein to the one containing 1g of protein. Therefore, it can be supposed that an increasing sample thickness helps to reduce the degradation rate, leading to more stable materials during exposure to the weathering agent. The sample containing 0.5 g of protein remains the sample with the highest tensile stress at break. It must be noted that the exposure to weathering agents is not the only factor of degradation for this type of materials, in fact also burial in soil test should be performed to achieve a complete break up, therefore this is in agreement with an only partial degradation as described in Figure 8."*

According with the addition of a new figure, Figure 8 (Moisture content and water solubility comparison) of the submitted manuscript become Figure 9 in the revised manuscript and Figure 9 (DSC diagrams) of the submitted manuscript become Figure 10, in the revised manuscript.

Comment #3:

The DSC results should provide much more information on the glass transition temperature. The authors did not mention this at all.

Response:

As suggested by the Reviewer the discussion about DSC results was strongly improved, and information about glass transition temperature was provided in section 3.2 as follows:

"*Tg is defined as the temperature at which the molecules binding forces are relaxed to allow large-scale molecular movement and can be detected as an endothermic flex of the DSC diagram. As shown in Figure 10 this flex can be attributed in the region 10-20°C and only insignificant difference can be detected from sample to sample, since the same quantity of plasticizer (glycerol), based on the protein amount, has been employed. The significant increasing of the flat region after Tg, due to increasing amount of protein, and therefore thickness, indicates an increasing rubbery region of the materials. Therefore, with the increasing thickness more flexible polymers can be obtained, as suggested by tensile strain results shown in Figure 7"*

Moreover, in the paragraph 2.4 Measurement a statement about Tg evaluation was provided:

"The glass transition temperature (Tg) was calculated as midpoint of the temperature range, bounded by the tangents to the two flat regions of the heat flow curve."

Response to Comments from the Reviewer #2

We would like to thank the reviewer for careful and thorough reading of this manuscript and for the thoughtful comments and constructive suggestions, which help to improve the quality of this manuscript. Our response follows (in italics the manuscript text, highlighted in yellow the new parts):

Comment #1:

Page 3, line 48: plasticizers might indeed reduce the polymer melting point, but more importantly, the glass transition temperature is reduced. Please correct.

Response:

As suggested by the Reviewer at page 3, line 48 "*melting*" was substituted with "*glass transition*".

Comment #2:
Page 4, line 20: reference is made to so-called ‘recent studies’. The cited references date form 1973, 2016 and 1998. Only one publication can be called ‘recent’

Response:

As suggested by the Reviewer references [54] and [56] were substituted with the following more recent publications:

[54] H.M.C. Azeredo, R. Morrugares-Carmona, N. Wellner, K. Cross, B. Bajka, K.W. Waldron, Food Chem. **2016**, 198, 101.

[56] P. González Seligra, C. Medina Jaramillo, L. Famá, S. Goyanes, Carbohydr. Polym. **2016**, 138,66.

Comment #3:
Page 5, line 44-47: It is written that that experiments were done on different amounts of protein while keeping the ratio’s with additives constant. I suggest writing that a study was performed on films with different thickness because that is essentially what it is …

Response:

As suggested by the Reviewer at page 5, line 44 the expression “*amount of protein*” was substituted with “*thickness*”

Comment #4:
Page 7, line 10: cause-effect relation between components and properties. Please mention the evaluated properties here

Response:
As suggested by the Reviewer at page 7, line 10 the phrase “*the proprieties of the obtained materials*” was substituted with “*the capability to form free standing materials*”

Comment #5:
Page 8, film preparation. Here I see a major problem. The treatment of the films seems to be not identical. Only the ones that contain citric acid (CA) received a heat treatment at 175°C for 5 min, whereas the other samples did not. In the end it will turn out that only the materials that did not contain CA received a score of 6 (the best score). This best score might be due to not having received a heat treatment rather than to the absence of CA. All samples need to receive the same heat treatment for a fair comparison. This likely implies a major change to the paper. Can the authors exclude protein degradation during the heat treatment?

Response:

The heating treatment at 175°C was performed only on samples containing CA because this is the procedure by which CA can be activated as crosslinker. As suggested by the Reviewer, in the discussion was not stressed enough that not receiving a score equal to 6 could be due to heating treatment that is strictly connected with the presence of CA in the mixture. Moreover, the authors performed further test treating the samples with the best scores at 175°C for 5 minutes. The results of these tests shown no modification of the specimens, due to the fact that nevertheless 175°C is a relative high temperature for organic materials, the time of 5 minutes is not enough to produce a significative protein degradation. For this reason, the DoE results discussion was improved as follow:
"It must be stressed that the heating treatment at 175°C, needed to activate the CA, could be a direct cause for not reaching the highest score of 6 due proteins degradation. Therefore, specific tests have been done heating in this same way the samples with the highest score, finding that no modification occurred and therefore lower score must be attributed only to the combination of GL and CA ".

Comment #6:
Page 10, line 24: the sample thickness was measured using digital micrometer. For samples that that are not equally thick over the cross-section of the measuring device you will always probe the maximum thickness of the sample. Such errors might lead to overestimations, in particular for thin samples where the film might be not homogenously thick (or even leave holes). Looking at the experimental results, this seems to be the case. Extrapolating the film thickness to zero protein content does not lead to a zero thickness, which means that the (average) thickness is overestimated at low protein contents and/or that the film does not cover the entire surface (see Figure 5). If you use overestimated film thicknesses in the density calculations, you will find underestimated densities. To my feeling any conclusion derived from the (apparent) density evolution should be reconsidered. Furthermore, the absence of error bars on the data in Figure 5 suggest that thickness measurements were very precise, but that might be related to the method and hide a systematic error. In any case, error bars should be added.

Response:

Thanks to the Reviewer for this important comment, following the Reviewer suggestion first of all error bars were added in Figure 5 both for thickness and density. These values, (average and error bars) were obtained measuring the samples in different point as stated in Page 10, line 25 to avoid errors due to irregular surfaces as much as possible. The authors replicate the tests, increasing the number of points of measure from 5 to 15, and the average values were confirmed. Therefore, the error bars added in Figure 5 are the ones obtained after 15 measures in different points. In paragraph 2.4 the authors added information about instrument sensibility ("*The sensibility of the instrument was 0.02mm"*) and about number of points of measure *("five"* was substituted with "*fifteen*"). Surely the results must be considered taking in account the error bars. Finally, the authors added a comment in the caption of Figure 5 "The *dashed lines are used to guide eyes only*"

Comment #7:

Page 10, DSC description: information on the sample holder is missing. It is important to know which pans were used and whether or not they were hermetically sealed (important for moisture retention). Furthermore, the measurements that are discussed are second heatings. A lot may happen to the samples (further crosslinking, degradation) during the first heating to 200°C of which nothing is reported. Therefore, the data in second heating are not relevant to the samples and their properties directly after film preparation. Please consider discussing the first heating runs. Finally, data should be presented after normalization to the sample mass for an adequate comparison.

Response:

Following the Reviewer suggestion in paragraph 2.4 Measurement more details were provided about the sample holder, sample measurement, and data normalization. In particular the two following sentences were added:

"A total of 5 ± 1 mg of each sample was loaded into a hermetic aluminum pan and an empty pan of the same material was used as reference during the same heat treatment"

"The obtained curves were normalized to the respective sample weight before comparison"

And the following sentence was erased to avoid any misunderstanding due to the fact that in Figure 10 the first heating was shown and discussed in the text.

"*~~Each sample was firstly heated from 25 °C to 200 °C at 20 °C min^{-1}, to erase the previous thermal history~~."*

Figure 10 was changed according to these statements.

Comment #8:

Page 12: the discussion on potential effects of glycerol and citric acid should contain statements on the possible reactions: i.e. the chemical reactions associated with the crosslinking or extension should be mentioned / explained. At what stage do they happen? During mixing, during the heat treatment? Is glycerol acting as reagent or medium for the chain linking reaction? Or is glycerol mainly acting as plasticizer? The potential reaction between glycerol and citric acid should also be considered. The solution pH is likely not in favor of such a direct reaction, but that might depend on the amount of water used, the ensuing heat treatment.. All of this should be taken into account in the discussion.

Response:

Following the Reviewer comments the discussion at page 12 was improved as follows and the associated literature reference were added:

"CA is an aliphatic polyfunctional bio-based raw material that contains two reactive primary carboxylic groups, one sterically hindered hydroxyl group and one less reactive tertiary carboxylic group [70]. In this work protein films were prepared at pH = 10 and thus, carboxylic groups in CA

were most probably in the form of carboxylates, that reacts with the functional groups N-terminal amine and forms an amide linkage, leading to the cross-link with protein after the heating treatment [71-72]. Indeed, nucleophilic substitution is the proposed mechanism for the reaction between protein and CA [73]. Therefore, the possible situation after the addition of GL could either be due to (a) GL reacting with CA, due to its three hydroxyl groups, it can partake in this reaction, competing with CA during crosslinking and reducing the proteins crosslinking with CA. (b) GL reacting with proteins, due to its low molecular weight that promotes the diffusion into proteins, reducing internal hydrogen bonding within the protein, due to its highly hydrophilic characteristics, thereby decreasing the internal forces and increasing the inter-molecular spacing, working as plasticizer and inhibiting the possible crosslinking with CA [74-75].”

[70]D. Hazarika, N. Karak, ACS Sustain. Chem. Eng. **2015**,3,2458.

[71] H. Xu, L. Shen, L. Xu, Y. Yang, Ind. Crops Prod. **2015**, 74, 234.

[72] K. Woods, GW Selling, J Appl Polym Sci. **2008**,109,2375.

[73] V. Froidevaux, C. Negrell, S. Caillol,JP. Pascault,B. Boutevin, Chem Rev. **2016**,116,14181.

[74] A. Awadhiya, D. Kumar, V. Vivek, Carbohydrate Polymers.**2016**, 151, 60.

[75] M. Martelli, S. Moore, G. Silva Paes, S. Gandolfo, C. Laurindo, Lwt-Food Sci Technol.**2006**, 39, 292.

Comment #9:
Page 12, line 29: the conclusion that GL should be maximized and CA should be minimize might be translated into: use as much plasticizer as possible and avoid high temperature treatments …

Response:
According with the Reviewer suggestion the following statement was added at Page 12 line 29 “*and consequently high temperature treatments”*

Comment #10:
Page 14, discussion related to Figure 6: all data are discussed in terms of overall crosslinking quality. I do not see why this would depend on the film thickness. In any case for such a claim, one should asses the crosslinking degree experimentally. Most likely the authors report engineering stresses, which rely on the initial sample dimensions. When necking is involved, dimensions change drastically and hence also the true stress. Yielding and the occurrence of necking besides on molecular parameters also depend on the presence of defects in the material (stress concentrations). Can the authors exclude (systematic) contributions from defects (local thickness differences or the presence of dust particles are to be considered as defects)?

Response:

Following the Reviewer comment part of the discussion about mechanical proprieties was rewritten taking in account the certain presence of defects, that play a key role for mechanical properties assessment, and avoiding the term "crosslinking", since this specific property was not measured experimentally. In the submitted manuscript the term crosslinking was used to describe a generic ability of the material structure to form a free-standing material with significant strength. In the present paper engineering stresses were compared and, due to the fact the all samples were affected by necking, the increasing of the true stresses due the reduced cross-section should be comparable as well.

For all these reasons the sentence "~~*the overall linking between the proteins chains is poor in these configurations"*~~ was substituted with: "*the microstructure configuration of these specimens is not suitable to perform a strong binding between proteins. In fact, below 0.5 g the lack in mechanical properties can be due to a too poor concentration of proteins avoiding the formation of a strength structure, on the opposite, over 0.5g the increment in thickness generates higher probability to find voids, microstructural defects and local thickness differences, acting as stress concentrators and leading to restrained mechanical properties of the overall material"* and the following statement was removed ~~*"The decrease of tensile stress at break with an increase in protein might be due to reduced chain linking density owing to lack of sufficient diffusion during the consolidating reactions, related to the process of obtainment of the specimen through casting technique."*~~

Comment #11:

Page 14, line 18-19: the lower value for the yield stress compared to the stress at break is interpreted as being due to 'brittle failure' of the material. Materials that can reach strains of 30% cannot be considered as brittle. Explain/reconsider. Higher stress values are readily explained by strain hardening due to molecular orientation. Actual stresses at break should even be higher than the reported values (recall my comment on the engineering stress versus true stress).

Response:

Following the Reviewer suggestions , the authors decided to better explain the term "brittle failure" that in the case of the present paper was used only as comparison between quite similar materials in terms of different degree of elastic/plastic behavior. Therefore, the following expression was removed: "~~*a likely brittle failure of this material*~~" and substituted with "*that, for this particular sample, the elastic component of the strain is strongly higher than the plastic one, leading to a less ductile material."*

Comment #12:

Page 14, 39-40: a comparison is made with literature materials based on proteins and glycerol. One should be more careful here. The authors use 46% plasticizer of which they claim that this corresponds well with plasticizer contents in the range 10-60 wt%. This is not true. For typical proteins increasing the plasticizer content from 15 to 30% makes the material Tg shift to below room temperature by which the material converts from a solid glass to a rubbery substance. Of course properties are in that case very different with the glasses being brittle and the rubbers being rather ductile. Judging from the mechanical data presented by the authors, clearly rubbery samples are in place. One should compare this material only with other rubbery protein based materials.

Response

One of the main novelties of the present paper is to employ animal proteins to produce biopolymers. Actually, a restrained amount of published data is available about this very specific source for biopolymer and the more important studies are focused on keratin, crayfish and albumen, as listed through the references of the present paper. Therefore, the authors decided to focus their attention on the comparison with these works about biopolymers based on animal proteins and employing a quantity of glycerol as much near possible to the quantity of the present paper, although as suggested by the Reviewer the mechanical behavior could change significantly in a so wide range of plasticizer (10-60%). Therefore, the authors decided to stress this fact in the discussion about mechanical properties and the following statement was added: "*These results are also consistent with the higher glass transition temperature reported for albumen and crayfish based materials with respect to the materials studied in the present paper, leading to a less ductile behavior at room temperature. It can be concluded that, at room temperature, BSF protein based materials are more ductile, and therefore with higher potential to be employed as biodegradable film, with respect to other animal protein based materials.*"

Comment #13:
Page 14, line 42: 'counting rotating' should be 'counter rotating'

Response:
Following the Reviewer suggestion, the expression "*counting rotating*" have been substituted with *"counter rotating"*

Comment #14:

Page 15, discussion related to Figure 8: it is claimed that the moisture content is a function of the sample thickness. This is not true: within experimental error, the moisture contents are equal

Response:

Following this Reviewer comment the discussion about the moisture content results, shown in Figure 9 of the revised manuscript, was improved. In particular, was stressed that the claim about the relation between moisture content and thickness is true only from a protein content equal to 1 g and above, considering the experimental error. The following highlighted statements were added to the manuscript:

"In particular the average value of moisture content (MC) increases moving from lower to higher quantity of protein employed, starting from 1g and above, and this trend is consistent with the marked hygroscopic behavior of the protein chains, so an increase of this property is expected with the increase of the amount of the proteins employed. It must be noted that below 1g of protein, considering the experimental error, the samples have the same moisture content. Therefore, the quantity equal to 1 g can be considered as a threshold for moisture content increment, due to increasing thickness, as the packing of the protein chain becomes less close, letting moisture be adsorbed more easily by the material."

Comment #15:

Page 16: the comparison with starch/LDPE systems is unfair because the LDPE is added to enhance the mechanical properties. A comparison should be made in terms of all relevant properties. It is unfair to compare each property with different other materials, selected based on that property being a weak point of the material the comparison is made with. For sure this does not make the material studied by the authors a superior material … A comparison between materials should be made in terms of all relevant properties.

Response:

Following the Reviewer comment the consideration about the comparison with starch/LDPE was deleted.

~~From this point of view, starch-based bioplastic is limited since they are mixed with hydrophobic LDPE, with negligible moisture content and water solubility~~

Comment #16:
Page 16, line 42-43: the difference in behavior is attributed to the proportional increase of the content of GL. This does not make any sense because the ratio protein/glycerol was kept constant in this series …

Response

Following this Reviewer comment together with comment #7 and #17 the discussion about DSC results was strongly improved, consequently the Figure 9 of the submitted manuscript was modified taking in account the sample mass normalization. Therefore, the statement highlighted by the Reviewer in this comment was deleted.

~~A different behavior could be observed only in the sample produced with 4 g of protein and a proportional increased content of GL, if compared with the other specimens analyzed.~~

Comment #17:

Page 16, Figure 9: Sample P4 displays an endothermic peak, which is notified (but poorly explained - see also previous remark) whereas the other sample display exothermic signals in this temperature range. These exothermic events are not discussed. Why? Anyway, discussing second heating runs is not very relevant. Please also see my remark 7 related to these DSC experiments.

Response

Following this Reviewer comment and also considering the comments #7 and # the figure reporting DSC data and discussion were strongly modified and improved considering the mass normalization, and more detailed information about the exothermic and endothermic signals were provided. In particular the following statement:

"~~*two small inflexions are detectable respectively in the range 10-20 °C and 75-90 °C depending on the specimen analyzed indicating that the material had fully denatured due to protein bioreactions"*~~

was substituted with:

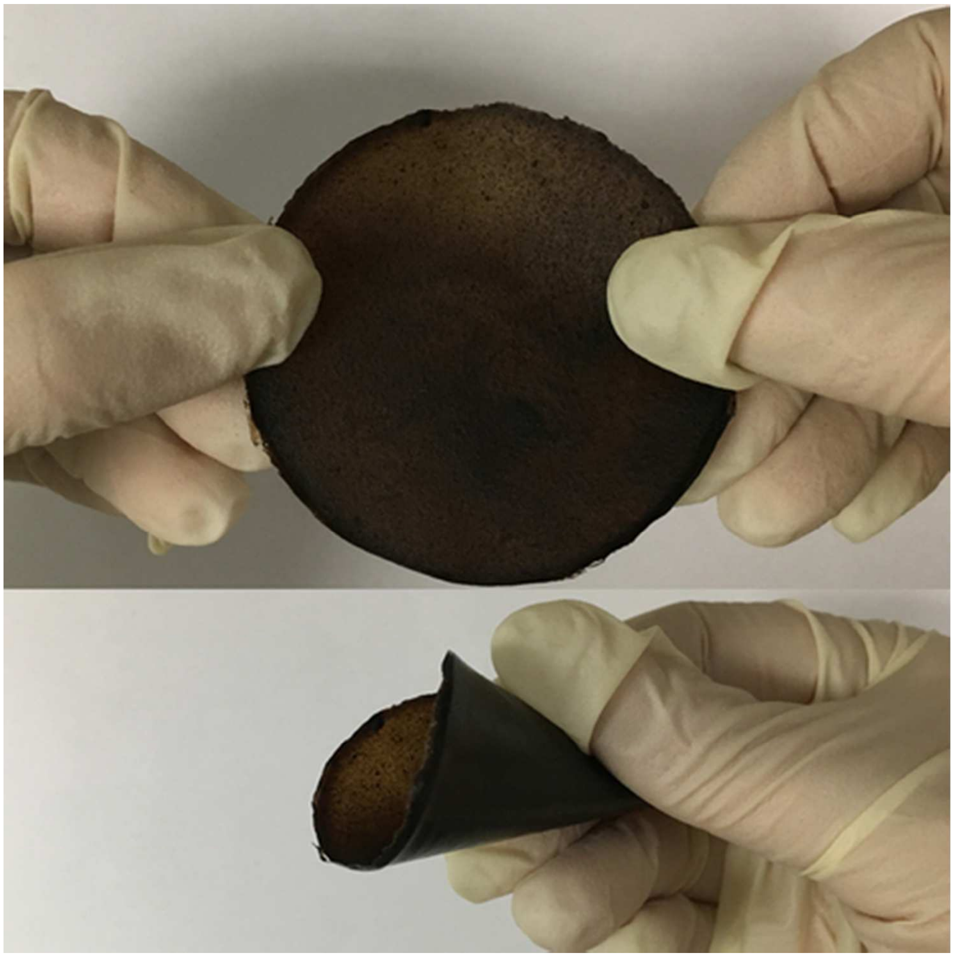
"*From -25°C to 10°C a sequence of weak exothermic peaks is detectable, indicating the presence of residual water due to the strong hydrophilic behavior of both proteins and glycerol. An endothermic inflexion can be detected in the region 10-20°C and attributed to the glass transition temperature."*

And therefore, the following statement was added about the peaks detected after Tg:

"Subsequently four transitions can be registered: a first low-temperature broad endothermic peak at about 80−100 °C likely due to evaporation of residual moisture of the protein, an evident *endothermic peak around 150-170 °C attributed to GL interaction with protein as already reported for other type of proteins plasticized with GL, a broad exothermic peak can be observed in the range 125-180°C (depending on the samples) as a result of a possible partial crystallization phenomenon and then finally >180°C the starting melt of the crystalline phase is registered.*[35,82]*"*

Finally, the following statement has been moved at page 14, into the discussion about DoE results:

"In fact, according with other studies, the addition of water in combination with GL, increases the polymer-water interactions to the detriment of polymer–polymer interactions.[81-82] *As it has been postulated, the effectiveness of plasticizers for bioplastics is highly dependent upon how they affect hydrogen bonding or hydrophobic interactions.*[83] "



40x40mm (300 x 300 DPI)

Rational design and characterization of bioplastics from *Hermetia illucens* prepupae proteins

Silvia Barbi[1*], Massimo Messori[2], Tiziano Manfredini[2], Martina Pini[3], Monia Montorsi[3]

*Corresponding author: silvia.barbi@unimore.it

[1]Interdepartmental center for applied research and services in advanced mechanics and motoring, University of Modena and Reggio Emilia, Via P. Vivarelli 10/1, Modena, 41125, Italy

[2]Department of Engineering "E. Ferrari", University of Modena and Reggio Emilia, Via P. Vivarelli 10/1, Modena, 41125, Italy

[3]Department of Science and Methods for Engineering, University of Modena and Reggio Emilia, Reggio Emilia, Via Amendola 2, 42122, Italy

Abstract. In this study proteins extracted from prepupae of *Hermetia illucens*, also known as black soldier fly, are investigated as promising base for a new type of bioplastics for agricultural purposes. Design of Experiments techniques are employed to perform a rational study on the effects of different combination of glycerol as plasticizer, citric acid as cross-linking agent and distilled water as solvent on the capability of proteins to form a free-standing film through casting technique, keeping as fixed the quantity of proteins. Glycerol shows interesting properties as plasticizer contributing to the formation of homogenous and free-standing film. Moreover, mechanical and thermal characterizations are performed to estimate the effect of increasing amounts of proteins on the final properties and thickness of the specimens. Proteins derived from *Hermetia illucens* can be successfully employed as base for bioplastics to be employed for agricultural purposes.

Keywords: biopolymers, recycling, proteins, waste, mechanical properties

1. Introduction

Bioplastics have attracted great attention in the last decades due to the urgent need to protect the environment from the large amount of plastic waste left daily in landfill or managed by waste-to-energy. The trend of the last years has seen the development and optimization of numerous bioplastics whose additional requirement is biodegradability which consists in the possibility that they can decompose in the environment relieving the pressure of waste management.[1] The development of always more innovative "eco-friendly" plastics represents a key challenge to reduce the non-degradable waste on planet and replenishing reserves of not renewable fossil fuels.[2] In 2016 the worldwide annual plastics production reached over 335 million tons where 60 million tons have been produced in Europe and this can be translated in trillions of dollars in terms of global economic returns.[3] An estimated 2–3 million tons of plastics are used each year in agricultural applications in order to increase the yield and quality of horticultural products.[4-6] Almost half of this amount is employed for the protection of the cultivations as soil mulching films, greenhouses, temporary coverings and low tunnels. Most of these applications are dominated using plastic based on low-density polyethylene (LDPE) due to its relatively good mechanical and optical properties, combined with a competitive cost.[7] The lifetime of LDPE films ranges from few months to 3–4 years depending on the thickness of the material and on the environmental condition near the soil.[8,9] After use, the recycling process is expensive and time-consuming due to too small plastic pieces remained after partial degradation, which often can be founded as pollutant in the soil.[10] Taking in account the direct impact of this type of pollutant on soil and cultivation and to overcome the disposal problems of conventional plastic, increasing the sustainability of agricultural exploit and cultivations, films based on biodegradable materials have to be considered as favorable alternative.[11–17] At the end of their lifetime, biodegradable plastic films can be composted directly in the soil, avoiding the formation of pollutant residues in the ground and furthermore producing an innovative and totally green source of sustainment for the plant production.[18] Several studies have been already presented about the formulation and employment of biodegradable films based on vegetables systems, such as soy, corn zein, wheat, cottonseed and sunflower.[19-24] The most relevant drawback highlighted by these studies is about sustainability, environmental and social impact of bio-based plastics from plant, that must be further investigated due to the potential exploitation of resources that can be employed for human nutrition.[25–30] Similar systems can also be produced by animal sources such as gelatin, collagen, keratin, feather quill, egg, or bacteria and this is exactly the context in which this study fits:[31-37] proteins extracted from insects grown on waste have been used to produce biopolymers, combining the positive effect due to the reduced impact on waste management with to the development of bio materials with potential degradability in the environment. It must be stressed that the growing interest about natural protein-based plastics is not only related to agriculture application but also to pharmaceutical and composite field of interests.[19, 38-42]

Protein-based polymers must be formulated introducing very specific additives, such as plasticizers and cross-linkers, to tailor the strong intermolecular interactions between protein chains, such as hydrogen bonding, electrostatic forces, hydrophobic bonding and disulfide cross-linking, to avoid excessive stiffness, brittleness and to increase the water absorption of the final material.[43] Generally, plasticizers are used to increase the flexibility and durability of the final material, but also to reduce the glass transition temperature and therefore to improve the processability, which often results in the decrease of the final cost of the polymers.[44, 45-48] The most common plasticizers used are polyols and mono-, di-, and oligosaccharides such as glycerol, that results as an eco-friendly additive already employed for cosmetic and pharmaceutic applications.[49]

Cross-linking agents, natural or synthetic, added to the plastic mixture act at the intermolecular chain level leading to improved mechanical, cohesion and barrier properties.[50-54] The most commonly used covalent cross-linking agents are glutaraldehyde, glyceraldehyde, formaldehyde, gossypol, and tannic and lactic acids, but their intrinsic toxicity can promote severe problems to human health even if the total amount of bio-based plastic in soil is quite low.[53] Only recent studies have shown that the citric acid addition promotes the cross-linking behavior for polysaccharide-based plastics.[54-56]

In this context the most innovative aspect of the present study concerns the employment of proteins deriving from the prepupae of *Hermetia illucens* Linnaeus 1758 (Diptera: Stratiomydae) better known as black soldier fly (BSF). BSF is considered an impressive source of nutrients like proteins, nowadays used as feed for animals, and fats, used in biodiesel production.[57-58] In addition, BSF is known as a safe waste bio-converter as it can grow on several types of substrates avoiding any disease transmission risk, increasing its potential relapse in the circular economy perspective.[59-62] In the present study, proteins derived by BSF prepupae, grown on substrate composed mostly by poultry manure, have been mixed with other components to obtain bioplastics for agricultural purpose. In this way, insects are used to process a waste and their fractionated prepupae to obtain proteins employed in a biopolymer with potential biodegradability, which can find applications in agricultural field. The biopolymer design has been conducted taking in account one of the most critical aspects, concerning the quantification of the correlation between each component in the formulation and the final properties of the material. It is well known that often the chemical-physical and mechanical performance of a material depends by the interactions among components working in synergic way into the mixture.[63] To overcome the critical issue regarding the One Factor at A Time approach (OFAT), statistical based approach such as Design of Experiments (DoE) can be successfully used to tailor specific properties of materials.[64] In this work, DoE has been employed to guarantee a rational planning of the experiments, which ensures the acquisition of the maximum significant information about the effect of the systematic and multi-variate changing of the amount of each component on the final properties of the material. For the specific purpose of this study, a screening design, mixing proteins derived by BSF with plasticizer (glycerol, GL), cross-linking agents (citric acid, CA) and distilled water (DI) as solvent, has been-set-up. From the experimental point of view the present work can be divided in two main parts; in the first part the type and content of additives, based on a constant amount of proteins, have been considered to obtain a homogenous and free-standing film. At the very beginning of this first part, the experimental plan has been set-up choosing the type and range of additives useful for the obtainment of biodegradable films by means of literature research.[49-54] Each experiment of the plan has been then carried out and evaluated through a panel test in which the quality of the obtained films has been estimated. No other response has been considered at this stage, due to the variability of the compactness of the samples that limit sometimes the evaluation of quantitative properties such as physical or mechanical ones. A mathematical model has been derived from the panel test data allowing to evaluate the most suitable combination of components used as starting point for the second step of this study. In this part of the study, films characterized by different thickness have been tested keeping as constant the selected best ratio between additives and protein content, in order to explore the physical and mechanical properties of the resulting materials. Figure 1 summarizes the work flow of this study.

2. Materials and Methods

As described in Figure 1, each stage of the investigation employed different methods and experimental techniques described in detail in the following sections.

2.1. Design of Experiments (DoE)

Rational approach, codified by DoE, was employed to obtain the highest amount of information using the minimum number of experiments, saving time and costs. In the first stage the effect of the amount of the mixture components used together with the protein fraction was investigated on the obtainment of plastic materials. Three factors were considered: DI, GL and CA, which were varied according to the ranges detailed in Table 1. The ranges as expressed in Table 1 were chosen in consideration of preliminary tests done on protein/GL and protein/CA samples employing different amounts of DI. In the case of protein/GL mixtures a very positive result was obtained in terms of ability to constitute a free-standing and flexible film, for samples containing at least 50 wt% of glycerol. On the opposite, protein/CA samples demonstrated good capacity to form a cohesive material but with loss of flexibility increasing the amount of CA. Therefore, a structural plan of experiments considering all the three factors was needed.

The reference employed was a constant quantity of protein equal to 1 g, therefore GL and CA were considered in weight percentages based on the fixed protein content. The other variables occurring in the process and not specifically considered in this study were kept constant during all the tests. The Design Expert 8.0 (Stat-Ease) code was used both to set-up experimental plan and to analyses the results. Due to the limited number of factors a full factorial design was selected. The number of experiments required for this experimental plan was equal to 2^3 (two level for three variables) with two replicates for each experiment for error estimation. Central points, considered as the arithmetic average of the factors levels, were added in quadruplicate to investigate the presence of curvature in the data analysis.[65] Finally, six more points were added in order to give a more detailed description of the behavior of the mixture without cross-linking agent (0% of CA). Therefore, a total of 26 experiments were planned (Table 2). All the experiments (runs) were carried out in a random way to avoid the presence of systematic errors.

Analysis of Variance (ANOVA) was used to point out the cause-effect relationship between components ratio and the capability to form free standing materials.[66] The main assumptions of the ANOVA are that each input factor is independent from each other, normally distributed, and that the variation of the response can be decomposed into different components to evaluate the effect of each factor, their interactions, and experimental error (or unexplained residual).[66] Through F-test, variation among all the samples, usually due to process difference or factor changes, is estimated as larger enough or not than the variation within samples obtained in same experimental conditions. The p-value is the statistical parameter used to evaluate the significance of the model and of each factor and represents the probability that the considered model or factor is significant ($p\text{-value}<0.05$) or not. The quality of the fit in terms of regression analysis and the predictive power of the model were evaluated by using the R^2 and Pred-R^2 respectively.[67] R^2 is the proportion of the variance in the dependent variables that is predictable from the independent variables and Pred-R^2 is analogous but associated with the predicted value.[67]

2.2. Materials

The BSF prepupae protein fraction were extracted following the procedure reported in a previous study and it is worth noting that the dry matter of BSF prepupae was composed approximately by 32wt% of protein.[62] The protein fraction was grinded through dry analytical mill (IKA, A10 basic) and sieved in order to ensure the homogeneity of the particle size, below 40 μm, with the aim to equalize the reactive surface available during the polymerization. Glycerol (GL, 99%, Sigma Aldrich), sodium hydroxide solution (1M, Sigma Aldrich), Sodium hypophosphite (>98%, Sigma Aldrich) and Citric Acid (CA, >99.5%, Sigma Aldrich) were employed as additives for protein-based film formulation.

2.3. Films Preparation

Protein-based films were prepared by mixing protein, GL and CA in DI, adjusted to pH 10 with NaOH (1N). The cross-linker, CA, and the catalyst sodium hypophosphite (50% on weight of CA) were mixed in DI along with GL. While a constant quantity equal to 1 g of proteins from BSF was employed for each experiment in Table 2, for the study of films with different thickness, a variable quantity of proteins (0.25, 0.5, 1, 2 and 4 g) was employed with a constant ratio of additives derived by the model obtained from the experimental plan in Table 2. Solutions of protein and additives were heated at 70 °C for 30 min and stirred at 200 rpm. Solutions were poured into aluminum dishes and allowed to cool and desiccate for 24 h at room temperature under fume hood. After drying, the samples containing CA were treated in oven at 175 °C for 5 min to get the cross-linking reaction. All films were conditioned in a controlled environment chamber at 25 °C and 50% relative humidity before characterization.

2.4. Measurement

The evaluation of the capability to constitute a free-standing plastic film was performed through a consensual panel and the scores obtained were recorded as responses and then analyzed by using statistical methods. The output of each experiment was evaluated taking into account the homogeneity of the sample after drying and therefore its compactness and detachability from the aluminum support. The detachability corresponds, in practice, to the free-standing capability of the obtained film. The panel test grouped all the 26 experiments in 6 categories and a score from 1 to 6 was attributed at each one category as shown in Figure 2 and Table 3. The panel test was carried out employing the judgements in blind of five people. Specifically, the classification number equal to 1 corresponds to the weakest quality (completely not homogenous), as well as the score equal to 6 corresponds to the sample with the highest quality (homogenous, completely compact and with good detachability). ANOVA was used to determine the significance of differences among samples and the level of confidence.

Concerning the second part of the study, in which samples with different thickness based on the best mixture derived from DoE results were characterized, the geometry was evaluated through measurement of thickness and diameter with a digital micrometer (Mitutoyo, YY-T1BD-2GYE) in fifteen different points, and the average value was taken as reference together with its calculated standard deviation. The sensibility of the instrument was 0.02mm.

Differential scanning calorimetry (DSC) measurements were performed from -40 °C to 200 °C under nitrogen (flow rate 50 ml min^{-1}) with a heating rate of 10 °C min^{-1} by a DSC TA 2010. A total of 5 ± 1 mg of each sample was loaded into a hermetic aluminum pan and an empty pan of the same material was used as reference during the same heat treatment. The obtained curves were normalized to the respective sample weights before comparison. ~~Each sample was firstly heated from 25 °C to 200 °C at 20 °C min^{-1}, to erase the previous thermal history~~. The glass transition temperature (Tg) was calculated as midpoint of the temperature range, bounded by the tangents to the two flat regions of the heat flow curve.

Tensile properties were measured on a DMA TA Q800 using a film tension set-up. Sizes of the rectangular specimens were 20 × 5 mm^2. The specimens were conditioned at standard conditions (25 °C; 50% RH) for 24 h before testing and were run in duplicates. The samples were then aligned and mounted in film clamps for the Q800 using a fixture designed for that purpose. Sample lengths were measured in the film stage assembly under an applied force of 0.05 N. The tensile properties of the plastic films were monitored as the films were elongated with an applied force which was ramped to l8 N at a rate of 0.05 N min^{-1} from 0.05 N. All samples were tested at room temperature.

The moisture content (MC) of the films was evaluated at 105 °C for the constant weight. Films were weighed (w_0) and then dried in an oven at 105 °C for 24 h. After this time, samples were reweighed (w_1) to determine their MC:[68]

$$\mathbf{MC}(\%) = \frac{(\mathbf{w_0}-\mathbf{w_1})}{\mathbf{w_0}} * \mathbf{100} \qquad (1)$$

For the water solubility (WS) measurement, the dried specimens were immersed in 200 mL of distilled water for 24 h and afterwards, the films were dried again in the oven at 105 °C for 24 h and weighed (w_2). WS values were calculated by the following equation: [69]

$$\mathbf{WS}(\%) = \frac{(\mathbf{w_1}-\mathbf{w_2})}{\mathbf{w_1}} * \mathbf{100} \qquad (2)$$

Al the weights were measured with analytical balance with sensibility of 0.00001g.

The degradation profile against time was evaluated on selected samples according with EN 17033:2018 and EN ISO 4892-2:2013 Method A cycle 1 for the measurement of the degradation due to artificial weathering. Rectangular specimens (50x15mm) were exposed in a closed chamber at irradiance of 0,51 W/(m^2 x nm), with fixed temperature equal to 38°C and relative humidity equal to 65% continuously for 500 hours. Deionized water was sprayed during the exposure with cycle of 18 minutes over 2 hours. Mechanical properties were measured after the exposure.

3.Results and Discussion

3.1 Experimental Plan

In the first part of this study the 26 experiments detailed in Table 2 have been realized and rated through the panel test to evaluate their quality, the results are reported in Table 4. A logarithmic transformation has been required to normalize the data and codify the hierarchy of the factors. The ANOVA results have been presented in Table 5 where the quantification of the significance of factors and their interactions, as well as curvature through central points, have been reported and significant parameters have been highlighted in grey color.

Model correlating the factors (in single or interaction) to the panel data evaluation is significant as confirmed by the F-value equal to 13.62 and by the p-value <0.0001, that means that probability of the data variation due to unknown factors is statistical irrelevant. Moreover, it is worth noting that only GL, CA and the ternary interaction DI-GL-CA are significant factors correlated to the quality of the obtained samples. Although the other factors are not significant they have been considered as part of the model to respect the model hierarchy. Moreover, it is relevant that the curvature is not significant and therefore the central points can be treated as additional data in the regression model, augmenting the design plan. R^2 and Pred-R^2 (Table 6) confirms the good fit of the data and a quite fair predictive power of the model, that is coherent with the screening design perspective used.

Estimation of the influence of the factors (in single or in interaction) on the quality of the samples in terms of homogeneity, compactness and freestanding capability, has been described in Figure 3 where the coefficients of the variables in the model are reported. The greater influence can be attributed to the interaction between GL and CA, in particular their effect impacts negatively the quality of the final bioplastic films: simultaneous increase of GL and CA leads to the decreasing of film quality. The other stronger effects derived by GL and CA as single factor and their influence on the final response is positive: it can be stated that, GL and CA play the main role on the properties of the final material but in an opposite way if they are combined or not. This can be explained considering that for this BSF protein-based system, the role of the cross-linking agent (CA) can be overlapped and partially substituted by the chain linking action played by GL in the materials polymerization. From the data analysis, it becomes evident that only GL or CA (separately) can be used with BSF protein to obtain a standing free bioplastic. On the contrary, if used together CA and GL lead to a too strong chains interconnection that results in a more brittle material with loss of compactness. CA is an aliphatic polyfunctional bio-based raw material that contains two reactive primary carboxylic groups, one sterically hindered hydroxyl group and one less reactive tertiary carboxylic group.[70] In this work protein films were prepared at pH = 10 and thus, carboxylic groups in CA were most probably in the form of carboxylates, that reacts with the functional groups N-terminal amine and forms an amide linkage, leading to the cross-link with protein after the heating treatment.[71-72] Indeed, nucleophilic substitution is the proposed mechanism for the reaction between protein and CA. [73] Therefore, the possible situation after the addition of GL could either be due to (a) GL reacting with CA, due to its three hydroxyl groups, it can partake in this reaction, competing with CA during crosslinking and reducing the proteins crosslinking with CA. (b) GL reacting with proteins, due to its low molecular weight that promotes the diffusion into proteins, reducing internal hydrogen bonding within the protein, due to its highly hydrophilic characteristics, thereby decreasing the internal forces and increasing the inter-molecular spacing, working as plasticizer and inhibiting the possible crosslinking with CA. [74-75]

About DI, even if this factor does not have a great impact on the response, its effect increases moving from single factor to the cubic interaction (DI-GL-CA) and this behavior is consistent with the role of solvent played by DI: an increase of this factor becomes relevant when the quantity of additives that have to be solubilized increases.

The numerical description of the model has been defined in terms of real factors and related calculated coefficients as shown in Equation 3.

$$\mathrm{Ln}\ (\text{Overall quality of the film}) = -2.93248 + 0.33956 * \mathrm{DI} + 5.09163 * \mathrm{GL} + 8.91549 * \mathrm{AC} - 0.37849 * \mathrm{DI} * \mathrm{GL} + 1.27052 * \mathrm{DI} * \mathrm{AC} - 14.44104 * \mathrm{GL} * \mathrm{AC} + 1.65685 * \mathrm{DI} * \mathrm{GL} * \mathrm{AC} \tag{3}$$

The contour plots (Figure 4) better explain the final quality of films as a function of the amount of each mixture components, in particular the DI amount has been kept as constant and equal to 7 g (a), 10 g (b) and 13 g (c). It can be concluded that highest quality of the final films can be achieved by using as much plasticizer as possible, avoiding the CA employment and consequently high temperature treatments (moving from blue to red region in the plot). It must be stressed that the heating treatment at 175°C, needed to activate the CA, could be a direct cause for not reaching the highest score of 6 due proteins degradation. Therefore, specific tests have been done heating in this same way the samples with the highest score, finding that no modification occurred and therefore lower score must be attributed only to the combination of GL and CA. Even if this trend can be observed for all the three contents of DI, it is worth noting that higher amount of DI promotes wider response surface fitting our purposes to obtain a high-quality material (red area). This result allows suggesting that DI doesn't act only as solvent but also support the polymerization process together with GL then limiting the needs of plasticizer in the mixture to obtain a good quality. In fact, according with other studies, the addition of water in combination with GL, increases the polymer-water interactions to the detriment of polymer–polymer interactions.[76-77] As it has been postulated, the effectiveness of plasticizers for bioplastics is highly dependent upon how they affect hydrogen bonding or hydrophobic interactions.[78] From the results obtained by the DoE analysis the best ratio of solvent and additives is the following: DI:13 g, GL: 85%, CA:0%.

3.2. Characterization of Bioplastic Films Based on Different Amount of Proteins

The effect of increasing content of BSF protein has been analyzed by keeping constant the DI at 13 g, the GL at 85wt% and avoiding CA presence in the mixture. All the prepared specimens (Table 7) have been found homogenous and free-standing confirming the statements previously discussed in 3.1, and the diameter measured for each specimen is 50.0 ± 0.5 mm. Obtained bioplastic films have been characterized in terms of physical properties such as thickness and apparent density as shown in Figure 5. An increase in thickness can be observed with the rising amount of proteins employed following an almost linear trend even if two different slopes around 1 g of protein can be recorded: below that content the thickness seems to approach an asymptote standing around at 0.2 mm. On the other hand, the density has a parabolic trend reaching a sort of plateau increasing the amount of protein over 1 g. It is worth noting that for both thickness and density a change of the respective trends can be observed at about 1 g of content: below this limit an important increase in apparent density can be noted whereas the increase in thickness is limited, while an opposite trend can be observed over the limit of 1 g of BSF protein. It can be supposed that most of the proteins employed to produce the specimens above 1 g contribute to increase the volume of the samples, instead below the same limit the added proteins fill the interchain empty spaces, increasing the apparent density of the final material.

Mechanical properties of plastic material provide an indication of expected polymer integrity under load conditions that would occur during processing, handling, usage, and storage. In Figure 6 and Figure 7 the mechanical properties of the five samples have been shown in terms of tensile stress at yield (σ_y), tensile stress at break (σ_b), tensile strain at yield (ε_y), tensile strain at break (ε_b). After yielding, on each specimen, a necking zone has been observed and the neck extended until final fracture. The higher strain at break is reached with the sample that is produced with 0.5 g of protein and associated with the thickness of 0.4 mm, as reported in Figure 5. Increasing or decreasing the content of proteins from 0.5 g causes a strong fall of the tensile stress at break, suggesting that ~~the overall linking between the proteins chains is poor in these configurations~~ the microstructure configuration of these specimens is not suitable to perform a strong binding between proteins. In fact, below 0.5 g the lack in mechanical properties can be due to a too poor concentration of proteins avoiding the formation of a strength structure, on the opposite, over 0.5g the increment in thickness generates higher probability to find voids, microstructural defects and local thickness differences, acting as stress concentrators and leading to restrained mechanical properties of the overall material. About the tensile stress at yield, it can be observed that the average value is below the average value of tensile stress at break for each analyzed specimen, even if the standard deviations suggests a partial overlap of the confidence ranges. This is true in particular for the sample employing the lower amount of proteins, equal to 0.25 g, indicating ~~a~~

~~likely brittle failure of this material~~ that, for this particular sample, the elastic component of the strain is strongly higher than the plastic one, leading to a less ductile material. The main trend about the strains recorded, as expressed in Figure 7, indicates an increasing of both yield and break strain, with the increase of protein content. Mechanical properties such as tensile stress and strain at yield and at break, are directly influenced by chain linking density and network structure of the resulting polymer. [79] ~~The decrease of tensile stress at break with an increase in protein might be due to reduced chain linking density owing to lack of sufficient diffusion during the consolidating reactions, related to the process of obtainment of the specimen through casting technique.~~ In Figure 6 and Figure 7 a detailed comparison has been shown with other films obtained from animal proteins, such as albumen, crayfish and keratin. [80-82] This comparison has been made taking in consideration animal proteins mixed with 10-60 wt% glycerol only, in strong similarity with the present study. The mixing of the constituents was made through counter rotating mixing and injection molding for the studies about albumen and crayfish, and through casting for the keratin-based study. The thickness of the compared specimens is <1mm, for all the studies considered, therefore the most reliable comparison should be done only with the BSF protein-based specimen containing < 1g of protein according with Figure 5. As compared to albumen-based bioplastic, BSF protein-based bioplastic, has relative higher tensile strength at break, not only in the average value considered, but also taking in account the standard deviation on the measurement, in particular if 0.5 g of protein is employed. [80] The same result is enhanced comparing crayfish-based and keratin-based bioplastic with BSF-protein bioplastic, in fact all the specimen investigated in this part of the present study show higher tensile strength at break. [81-82] An overall stronger increase of strain at break can be evaluated if protein from BSF are employed with respect to albumen-based and crayfish-based bioplastic as shown in Figure 7.[81-82] These results are also consistent with the higher glass transition temperature reported for albumen and crayfish based materials with respect to the materials studied in the present paper, leading to a less ductile behavior at room temperature. It can be concluded that, at room temperature, BSF protein based materials are more ductile, and therefore with higher potential to be employed as biodegradable film, with respect to other animal protein based materials. It is worth noting that BSF bioplastic tested in the present study, in strong similarity with others animal protein-based films reported in literature, fail at low stress and small strain, indicating that the polymers had low elasticity if compared with starch-based bioplastic already available on the market, that generally shown tensile stress at break around 20 MPa and strain at break over 200%.[83] Nevertheless, taking in account the tensile resistance and elongation, BSF protein-based biopolymer are the more promising alternative to vegetable-based biopolymer among the animal-based ones.

The degradation profile against time of three selected samples have been measured as shown in Figure 8. Sample containing 0.5 g of protein has been chosen due the highest tensile stress at break measured, instead the other two samples have been tested as comparison of two opposite situation, both representative of a decreasing tensile stress at break, as shown in Figure 7 and previously discussed. As expected, a decreasing of the tensile stress at break with the increasing of the time under exposure to accelerated weathering has been measured for all the samples investigated, with very similar rate of degradation. Nevertheless, a decreasing rate of degradation can be observed moving from the sample containing 0.25g of protein to the one containing 1g of protein. Therefore, it can be supposed that an increasing sample thickness helps to reduce the degradation rate, leading to more stable materials during exposure to the weathering agent. The sample containing 0.5 g of protein remains the sample with the highest tensile stress at break. It must be noted that the exposure to weathering agents is not the only factor of degradation for this type of materials, in fact also burial in soil test should be performed to achieve a complete break up, therefore this is in agreement with an only partial degradation as described in Figure 8.

As shown in Figure 9, both the moisture content and water solubility strongly depend by the quantity of protein employed to produce the specimens, according to the high hydrophilic behavior of proteins. In particular, the average value of moisture content (MC) increases moving from lower to higher quantity of protein employed, starting from 1g and above, and this trend is consistent with the marked hygroscopic behavior of the protein chains, so an increase of this property is expected with the increase of the amount of the proteins employed. It must be noted that below 1g of protein, considering the experimental error, the samples have the same moisture content. Therefore, the quantity equal to 1 g can be considered as a threshold for moisture content increment, due to increasing thickness, as the packing of the protein chain becomes less close, letting moisture be adsorbed more easily by the material. The water solubility (WS), also known as total soluble matter (TSM), is therefore strongly favored for the specimens with lower thickness and on the opposite when the thickness is increased. As demonstrated in similar studies, for specimens with the same thickness, the WS parameter is related with the content of highly hydrophilic components or to moisture content as well as to proteins that are not strongly associated to the network structure. [80] For this reason in Figure 9 a detailed comparison has been shown with the same albumen-based film employed for the comparison of the mechanical properties. Also, in this case the comparison must be done taking in account the samples containing <1g of BSF-protein to compare film with the same thickness. [80] As compared to albumen-based bioplastic, BSF protein-based bioplastic, has relative higher WS and lower MC, not only in the average values considered, but also taking in account the standard deviations on the measurements. From these results it can be noted that BSF-protein bioplastic has a higher content of protein not strongly associated to the network , and therefore more free to be released in soil, as possible soil fertilizer during the plastic degradation for agricultural purposes. It must be noted that the water solubility is therefore one of the base properties to evaluate for a compostable material, and high value of this parameters suggest a marked inclination to waste disposal as beneficial compost material in soil. [13,69] Therefore the BSF-protein based bioplastics studied in the present study are more suitable as materials for agricultural purposes, with respect to albumen-based bioplastic due their enhanced capability to degrade in water. ~~From this point of view, starch-based bioplastic is limited since they are mixed with hydrophobic LDPE, with negligible moisture content and water solubility~~.

The experimental results of DSC analysis reported in Figure 10 show that about the samples obtained employing from 0.25 to 2 g of proteins the recorded curves are almost similar in shape and peak areas: ~~two small inflexions are detectable respectively in the range 10-20 °C and 75-90 °C depending on the specimen analyzed indicating that the material had fully denatured due to protein bioreactions~~. ~~A different behavior could be observed only in the sample produced with 4 g of protein and a proportional increased content of GL, if compared with the other specimens analyzed.~~ ~~For this sample an evident endothermic peak around 150 °C can be detected and attributed to GL interaction with protein as already reported for other type of proteins plasticized with GL.~~[35,75] From -25°C to 10°C a sequence of weak exothermic peaks is detectable, indicating the presence of residual water due to the strong hydrophilic behavior of both proteins and glycerol. An endothermic inflexion can be detected in the region 10-20°C and attributed to the glass transition temperature. Tg is defined as the temperature at which the molecules binding forces are relaxed to allow large-scale molecular movement and can be detected as an endothermic flex of the DSC diagram. As shown in Figure 10 this flex can be attributed in the region 10-20°C and only insignificant difference can be detected from sample to sample, since the same quantity of plasticizer (glycerol), based on the protein amount, has been employed. The significant increasing of the flat region after Tg, due to increasing amount of protein, and therefore thickness, indicates an increasing rubbery region of the materials. Therefore, with the increasing thickness more flexible polymers can be obtained, as suggested by tensile strain results shown in Figure 7.

Subsequently four transitions can be registered: a first low-temperature broad endothermic peak at about 80−100 °C likely due to evaporation of residual moisture of the protein, an evident endothermic peak around 150-170 °C attributed to GL interaction with protein as already reported for other type of proteins plasticized with GL, a broad exothermic peak can be observed in the range 125-180°C (depending on the samples) as a result of a possible partial crystallization phenomenon and then finally >180°C the starting melt of the crystalline phase is registered. ~~In fact, according with other studies, the addition of water in combination with GL, increases the polymer-water interactions to the detriment of polymer–polymer interactions.[81-82] As it has been postulated, the effectiveness of plasticizers for bioplastics is highly dependent upon how they affect hydrogen bonding or hydrophobic interactions.[83]~~

4. Conclusions

Proteins isolated by extraction from Black Soldier Flies prepupae and dissolved in water with addition of GL shown highly functional potential as a polymer matrix for film formation. The application of a Design of Experiments approach allowed investigating the influence of environmental-friendly plasticizer and cross-linking agents onto physical properties of protein-based materials in a systematic way, by clearly identifying composition regions where the formation of free-standing film is maximized and where a synergic effect can be observed between plasticizer and cross-linking agent. The addition of GL as plasticizer caused noticeable and positive changes in film structure leading to a significant reinforcement of the polymeric matrix. On the opposite, the addition of CA as green cross-linking agent has not beneficial effects on film forming due probably a functional overlap with GL, and for this reason CA should be completely avoided in bioplastic films formulation based on proteins from BSF. However, the mechanical and moisture related properties have been found to be mainly dependent on the protein content and the best properties in terms of tensile strength have been found employing 0.5 g of protein, leading also to a material with a quite restrained thickness. Nevertheless, improvements should be made in composition by including further mixture components with the aim to increase tensile stresses and strains to achieve trend comparable to other bioplastic materials already available on the market, this study confirms that proteins extracted from BSF could be used to obtain bioplastic films that should be promising for application in certain types of bio-compostable plastics, with the add value to use proteins deriving by the digestion of waste by insect and then contributing to the circular economy perspective.

Acknowledgements: This work was done in the framework of the project “ValoriBio” co-financed by 2014-2020 POR FESR, Emilia-Romagna Region, Italy, DGR 774/2015 – CUP E42I15000110009. The authors declare that there is no conflict of interests.

References

[1] L. Jiang, J. Zhang, in Applied Plastics Engineering Handbook: Processing, Materials, and Applications, Vol. 1 (Ed. M.Kutz), Elsevier, London, England, 2016, p.127.

[2] S.N. Bharti, G.S. Swetha, J Pet Environ Biotechnol. **2016**, 07, 2.

[3]http://www.plasticseurope.org/application/files/5715/1717/4180/Plastics_the_facts_2017_FINA L_for_website_one_page.pdf (accessed 22/05/2018) .

[4] www.ibaw.org (accessed 22/05/2018).

[5] www.plasticulture.com (accessed 22/05/2018).

[6] P.A. Dilara, D. Briassoulis, J. Agric. Eng. Res. **2000**, 76, 309.

[7] D. Briassoulis D. Polym. Degrad. Stab. **2005**, 88, 489.

[8] P. Desriac, Plasticulture. **1991**, 89, 9.

[9] J. Lemaire, Plasticulture. **1993**, 97, 17.

[10] G. Scott, Polymers and the environment. RSC paperbacks, London, England **1999**.

[11] D. Briassoulis, J. Polym. Environ. **2004**,12,65.

[12] R.Chandra, Prog. Polym. Sci. **1998**, 23,1273.

[13] E. Chiellini, P. Cinelli, A. Corti, E.R. Kenawy, Polym. Degrad. Stab. **2001**, 73,549.

[14] J.W. Doran, Agric. Ecosyst. Environ., **2002**, 88,119.

[15] D.L. Kaplan, J.M. Mayer, D. Ball, J. McCassie, S. Stenhouse, Biodegradable polymers and packaging. Technomic Publishing Co., Lancaster, England **1993**.

[16] A. Krzan, S. Hemjinda, S. Miertus, A. Corti, E. Chiellini, Polym. Degrad. Stab, **2006**, 91, 2819.

[17] M.Malinconico, B. Immirzi, S. Massenti, F.P. La Mantia, P. Mormile, L. Petti, J. Mater. Sci. **2002**, 37, 4973.

[18] G. Scott, Polym. Degrad. Stab, **2000**, 68, 1

[19] H. Tian, J. Compos. Mater. **2012**, 46, 427.

[20] L. Di Gioia, B. Cuq, S. Guilbert. Int. J. Biol. Macromol. **1999**, 24, 341.

[21] Y. Song, Q. Zheng, J. Cereal Sci. **2009**, 50, 376.

[22] **S.** Domenek, M.H. Morel, A. Redl, S. Guilbert, Macromol. Symp. **2003**,197,181

[23] H.B. Yue, C.D. Cui, P.S. Shuttleworth, J.H. Clark, Green Chem. **2012**, 14, 2009.

[24] C. Verbeek, L. van den Berg, Macromol. Mater. Eng. **2010**, 295, 10

[25] R.S. Tocchetto, R.S. Benson, M. Dever, J. Polym. Environ, **2002**, 9, 57.

[26] D. Briassoulis, Biosyst. Eng. **2004**, 87, 209.

[27] D. Briassoulis, Polym. Degrad. Stab, **2006**, 91, 1256.

[28] D. Briassoulis, J. Polym. Environ. **2006**, 14, 289.

[29] D. Briassoulis, Polym. Degrad. Stab, **2007**, 92, 1115.

[30] G. Scarascia-Mugnozza, E. Schettini, G. Vox, Biosyst. Eng. **2004**, 87, 479.

[31] G. Scarascia-Mugnozza, E. Schettini, G. Vox, M. Malinconico, B. Immirzi, S. Pagliara S. Polym. Degrad. Stab, **2006**, 91, 2801.

[32] V.Madison, J. Schellman, Biopolymers, **1972**, 11,1041.

[33] C.J.R. Verbeek, L.E. van den Berg. J. Polym. Environ. **2011**, 19, 1.

[34] A. Denis, N. Brambati, B. Dessauvages, S. Guedj, C. Ridoux, N. Meffre, Food Hydrocoll.**2008**, 22,989.

[35] A. Ullah, T. Vasanthan, D. Bressler, A.L. Elias, J. Wu, Biomacromolecules, **2011**, 12, 3826.

[36] A. Jerez, P. Partal, I. Martinez, C. Gallegos, A. Guerrero, J. Food Eng. **2007,** 82, 608.

[37] T. Mekonnen, P. Mussone, N. El-Thaher, P.Y.K. Choi, D.C. Bressler, Macromol. Mater. Eng. **2013**, 298,1294.

[38] N. El-Thaher, T. Mekonnen , P. Mussone, D. Bressler, P. Choi, Ind. Eng. Chem. Res. **2013**, 52, 4987.

[39] K. Dangaran, P.M. Tomasula, P. Qi, in Edible Films and Coatings for Food Applications, (Eds M. Embuscado, K.C. Huber) Springer, New York, USA 2009.

[40] A.O. Elzoghby, W.S. Abo El-Fotoh, N.A. Elgindy, J. Control. Release, **2011**, 153, 206.

[41] S. Khosravi, P. Nordqvist, F. Khabbaz, M. Johansson, Ind. Crop. Prod. **2011**, 34, 1509.

[42] Z. Peles, M. Zilberman, Acta Biomater. **2012**, 8, 209.

[43] R. Nakamura, A.N. Netravali, A.B. Morgan, M.R. Nyden, J.W. Gilman, Fire Mater. **2013**, 37,75.

[44] R. Sothornvit, J.M. Krochta, J. Food Eng. **2001**, 50, 149.

[45] S. Varughese, D.K. Tripathy, J. Elastomers Plast. **1993**, 25,343.

[46] L.H. Sperling, Introduction to physical polymer science. Wiley, Hoboken, NJ, USA **2005**.

[47] P. De Groote, J. Devaux, P. Godard, J. Polym. Sci. Part A Polym. Chem. **2002**, 40, 2208.

[48] E. Snejdrova, M. Dittrich, in Recent Advances in Plasticizers (ed. M.Luquam) Intech, London, UK 2012.

[49] N. Ljungberg, B. Wesslén, Polymer, **2003**, 44 ,7679.

[50] B. Cuq, N. Gontard, S. Guilbert, Cereal Chem. **1998**, 75,1.

[51] S.Guilbert, in Food Packaging and Preservation (ed M. Mathlouthi), Elsevier, New York, USA 1986.

[52] C. Remunan-Lopez, R. Bodmeier, J. Control. Release, **1997**, 44, 215.

[53] C. Marquie, C. Aymard, J.L. Cuq, S. Guilbert, J. Agric. Food Chem. **1995**, 43, 2762.

[54] H.M.C. Azeredo, R. Morrugares-Carmona, N. Wellner, K. Cross, B. Bajka, K.W. Waldron, Food Chem. **2016**, 198, 101.

[55] J. Uranga, I. Leceta, A. Etxabide, P. Guerrero, K. de la Caba, Eur. Polym. J**.** **2016**, 78,82.

[56] P. González Seligra, C. Medina Jaramillo, L. Famá, S. Goyanes, Carbohydr. Polym. **2016**, 138, 66.

[57] G.L. Newton, C.V. Booram, R.W. Barker, O.M. Hale, J. Anim. Sci.**1977**, 44, 395.

[58] V.C. Cummins, S.D. Rawles, K.R. Thompson, A. Velasquez, Y. Kobayashi, J. Hager, C.D. Webster, Aquaculture. **2017**, 473, 337.

[59] S. Diener, N.M. Studt Solano, F.R. Gutiérrez, C. Zurbrügg, K. Tockner, Waste Biomass Valorisation, **2011**, 2, 357.

[60] D.C. Sheppard, J.K. Tomberlin, J.A. Joyce, B.C. Kiser, S.M. Sumner, J. Med. Entomol. **2002**, 39, 695.

[61] T. Spranghers, M. Ottoboni, C. Klootwijk, A. Ovyn, S. Deboosere, B. De Meulenaer, S. De Smet, J. Sci. Food Agric. **2017**, 97, 2594.

[62] A. Caligiani, A. Marseglia,G. Leni,S. Baldassarre, L. Maistrello, A. Dossena,S. Sforza, Food Res. Int. **2018**, 105, 812.

[63] M.K. Fehri, C. Mugoni¸P. Cinelli, I. Anguillesi, M.B. Coltelli, S. Fiori, M. Montorsi, A. Lazzeri, Express Polym. Lett., **2016**, 10, 274

[64] D.C. Montgomery, Design and Analysis of Experiments. John Wiley & Sons Inc**,** New York, USA 2012.

[65] G.E.P. Box, W.J. Hunter, J.S. Hunter, Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building, Wiley Series in Probability and Mathematical Statistics, Hoboken, NJ, USA 1978.

[66] R. Leardi, Anal. Chim. Acta, **2009**, 652, 161.

[67] C.C. Colbourn, J.H. Dinitz, Handbook of Combinatorial Designs. Chapman and Hall/CRC, Boca Raton, USA, **2006**.

[68] Association of Official Analytical Chemists, Official Methods of Analysis of the AOAC International, AOAC, Arlington, USA **1995**.

[69] N. Gontard, C. Duchez, J. Cuq, S. Guilbert, Int. J. Food Sci. Technol.**1994**, 29, 39.

[70]D. Hazarika, N. Karak, ACS Sustain. Chem. Eng. **2015**,3,2458.

[71] H. Xu, L. Shen, L. Xu, Y. Yang, Ind. Crops Prod. **2015**, 74, 234.

[72] K. Woods, GW Selling, J Appl Polym Sci. **2008**,109,2375.

[73] V. Froidevaux, C. Negrell, S. Caillol,JP. Pascault,B. Boutevin, Chem Rev. **2016**,116,14181.

[74] A. Awadhiya, D. Kumar, V. Vivek, Carbohydrate Polymers.**2016**, 151, 60

[75] M. Martelli, S. Moore, G. Silva Paes, S. Gandolfo, C. Laurindo, Lwt-Food Sci Technol.**2006**, 39, 292.

[76] P. Chen, L. Zhang, Macromol. Biosci. **2005**, 5, 237.

[77] N.L.M. Robertson, J.A. Nychka, K. Alemaskin, J.D.J. Wolodko, Appl. Polym. Science. **2013**, 130, 969.

[78] J. Lunt, A.L. Shafer, Polylactic Acid Polymers from Corn: Applications in the Textiles Industry, Cargill Dow Polymers, Minnetonka, USA **2001**.

[79] M. Ganglani, S.H. Carr, J.M. Torkelson, Polymer, **2002**, 43**,** 2747.

[80] M. Félix, A. Romero, A. Guerrero, J. Food Eng. **2014**, 125,7.

[81] M.Felix, A. Romero, F. Cordobes, A. Guerrero, J. Sci. Food Agric. **2014**, 95, 679.

[82] N. Ramakrishnan, S. Sharma, A. Gupta, B.Y. Alashwal, Int. J. Biol. Macromol. **2018**, 111, 352.

[83] M. Niaounakis, Biopolymers : applications and trends, Elsevier, Oxford, UK **2015**.

List of Figures

Figure 1 – Workflow of the present study.

Figure 2 – Panel test evaluation.

Figure 3 – Model coefficients.

Figure 4 – Contour plots of the mixture with DI=7 g (a); DI=10 g (b); DI=13 g (c).

Figure 5 – Measured thickness and calculated density in function of the amount of protein. The dashed lines are used to guide eyes only.

Figure 6 – Tensile stress at yield (σ_y) and tensile stress at break (σ_b) of bioplastic films tested in the present study and based on different amounts of BSF protein. Comparison with data from literature about albumen based bioplastic [77], crayfish based bioplastic [78] and keratin based bioplastic.[79]

Figure 7 – Tensile strain at yield (ε_y), tensile strain at break (ε_b) of bioplastic films tested in the present study and based on different amounts of BSF protein. Comparison with data from literature about albumen based bioplastic [77] and crayfish based bioplastic. [78]

Figure 8 – Profile degradation of tensile stress at break Vs Time

Figure 9 – Moisture content (MC) and water solubility (WS) of bioplastic films tested in the present study and based on different amounts of BSF protein. Comparison with data from literature about albumen based bioplastic. [77]

Figure 10 –DSC thermograms of bioplastic films based on different amount of proteins.

List of Tables

Table1 - Factors and their levels

Factor	Low Level	High Level	Unit
DI	7.00	13.00	g
GL	50	100	wt%
CA	0	40	wt%

Table 2 - Experimental plan

RUN	DI (g)	GL (wt%)	CA (wt%)
1	7.00	50	10
2	13.00	100	40
3	7.00	100	40
4	10.00	75	25
5	10.00	75	25
6	13.00	50	10
7	7.00	50	40
8	7.00	100	10
9	13.00	50	10
10	13.00	50	40
11	7.00	100	40
12	13.00	100	40
13	10.00	75	25
14	7.00	50	40
15	7.00	50	10
16	13.00	100	10
17	7.00	100	10
18	13.00	100	10
19	10.00	75	25
20	13.00	50	40
21	7.00	50	0
22	13.00	50	0
23	10.00	50	0
24	7.00	100	0
25	13.00	200	0
26	10.00	100	0

Table 3 – Panel test categories and scores

JUDGEMENT	SCORE
Completely not homogeneous	1
Homogenous but not compact	2
Homogenous and partially compact	3
Homogenous and almost completely compact	4
Homogenous, completely compact with poor detachability	5
Homogenous, completely compact and with good detachability	6

Table 4 – Panel test evaluation

RUN	1	2	3	4	5	6	7	8	9	10	11	12	13
Evaluation	2	4	2	3	3	2	1	5	2	1	2	4	3
RUN	14	15	16	17	18	19	20	21	22	23	24	25	26
Evaluation	1	2	5	5	5	3	1	1	6	6	6	6	6

Table 5 – ANOVA analysis

Source	F Value	p-value Prob > F	
Model	13.62	< 0.0001	Significant
DI	4.18	0.0557	Not significant
GL	45.33	< 0.0001	Significant
CA	3.01	< 0.0001	Significant
DI-GL	0.15	0.7002	Not significant
DI-CA	0.040	0.842	Not significant
GL-CA	2.40	0.1387	Not significant
DI-GL-CA	12.73	0.0022	Significant
Curvature	2.34	0.1443	Not significant

Table 6 – Fitting parameters of the model

R^2	0.8412
Pred R^2	0.6721

Table 7 – Composition of bioplastic films based on different amount of proteins.

Protein (g)	DI (g)	GL (g)	CA(g)
0.25	3.25	0.21	0
0.50	6.50	0.42	0
1.00	13.00	0.85	0
2.00	26.00	1.70	0
4.00	52.00	3.40	0

Figure 1 – Workflow of the present study.

70x61mm (300 x 300 DPI)

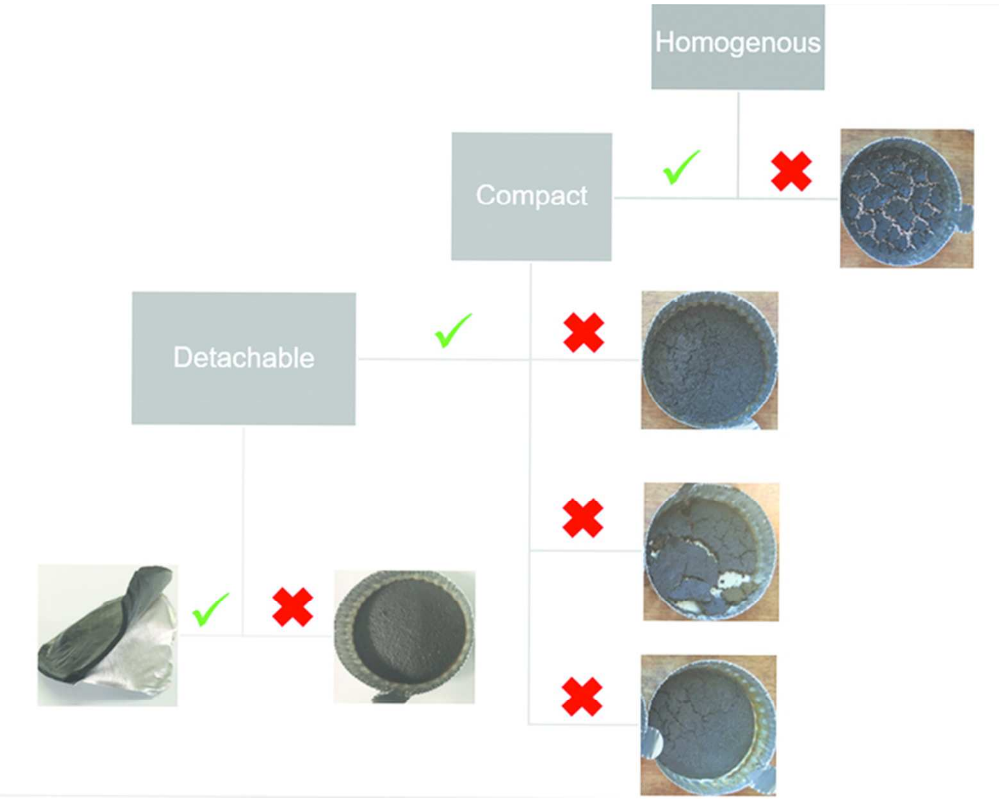


Figure 2 – Panel test evaluation.

63x50mm (300 x 300 DPI)

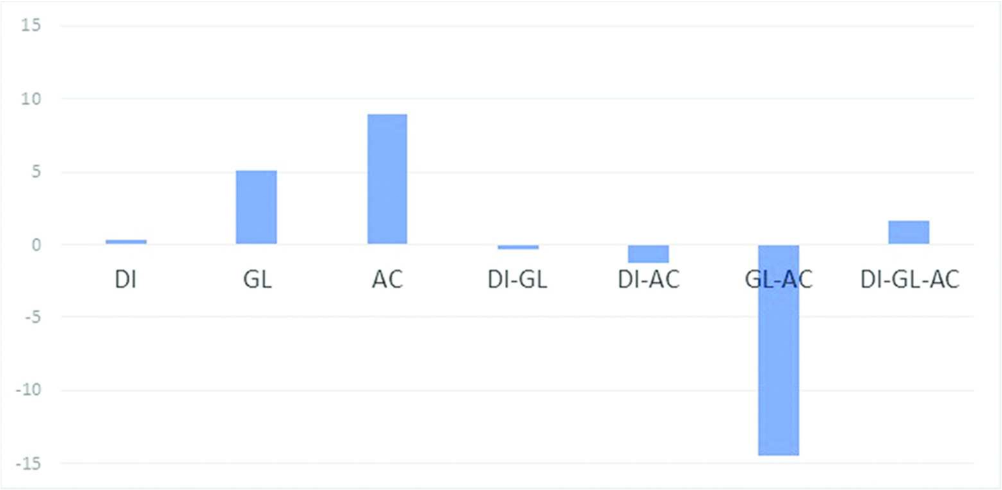


Figure 3 – Model coefficients

75x36mm (300 x 300 DPI)

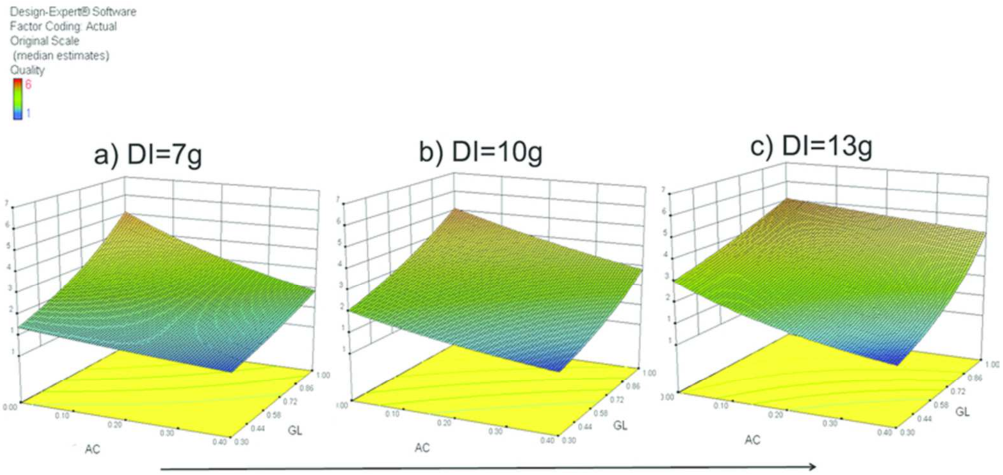


Figure 4 – Contour plots of the mixture with DI=7 g (a); DI=10 g (b); DI=13 g (c).

70x33mm (300 x 300 DPI)

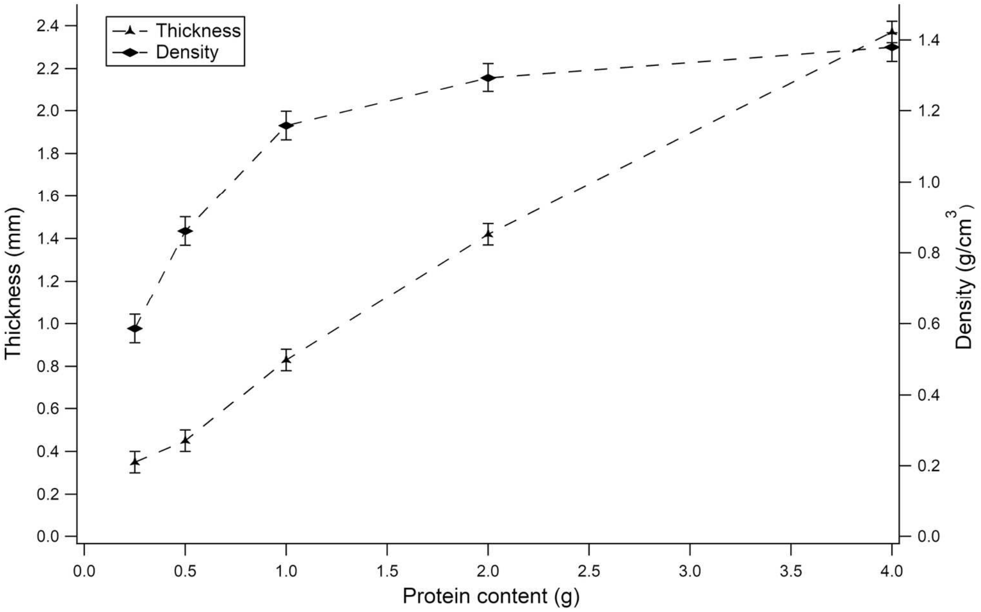
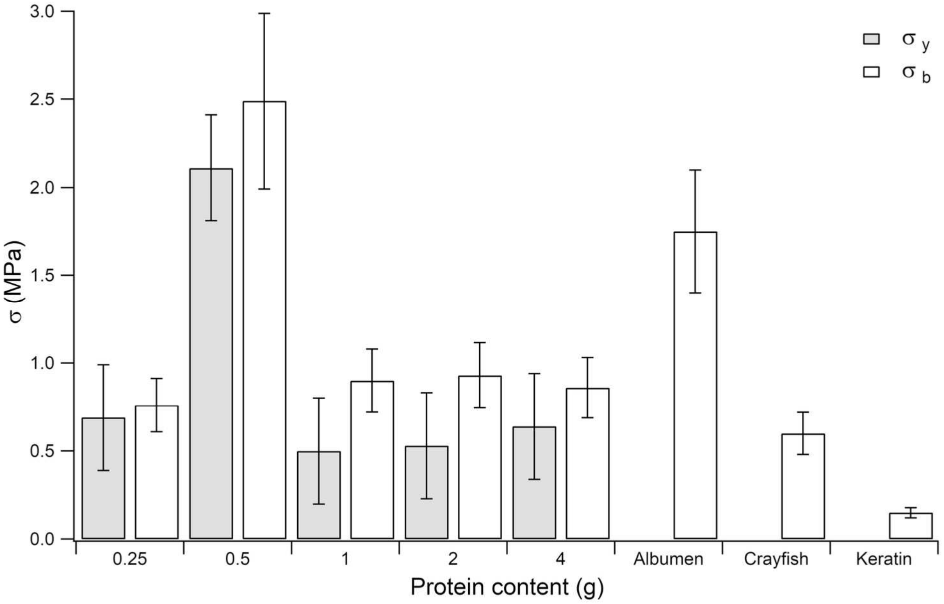


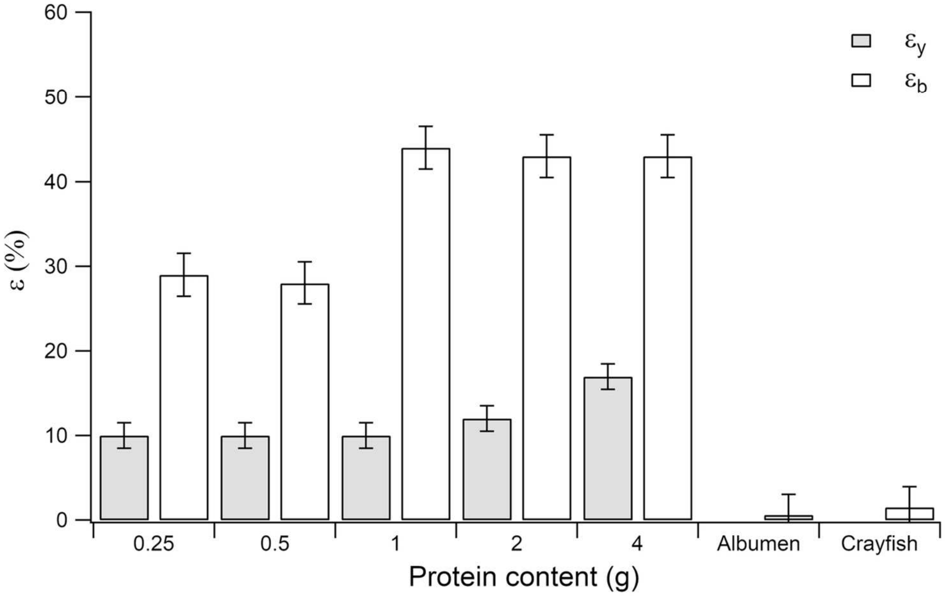
Figure 5 – Measured thickness and calculated density in function of the amount of protein. The dashed lines are used to guide eyes only.

162x101mm (300 x 300 DPI)



Tensile stress at yield (σy) and tensile stress at break (σb) of bioplastic films tested in the present study and based on different amounts of BSF protein. Comparison with data from literature about albumen based bioplastic [77], crayfish based bioplastic [78] and keratin based bioplastic.[79]

148x92mm (300 x 300 DPI)



Tensile strain at yield (εy), tensile strain at break (εb) of bioplastic films tested in the present study and based on different amounts of BSF protein. Comparison with data from literature about albumen based bioplastic [77] and crayfish based bioplastic. [78]

124x76mm (300 x 300 DPI)

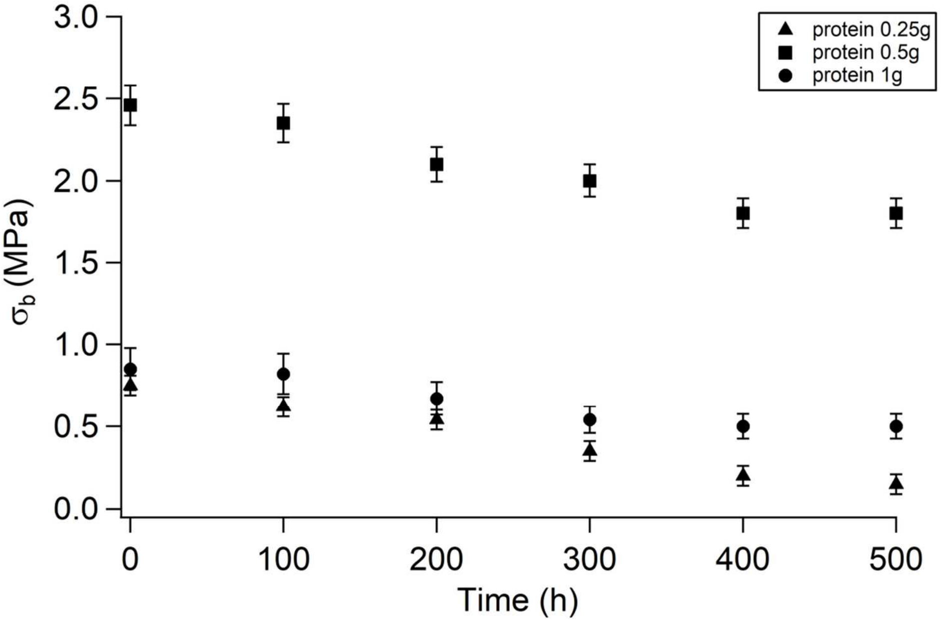


Figure 8 – Profile degradation of tensile stress at break Vs Time

110x70mm (300 x 300 DPI)

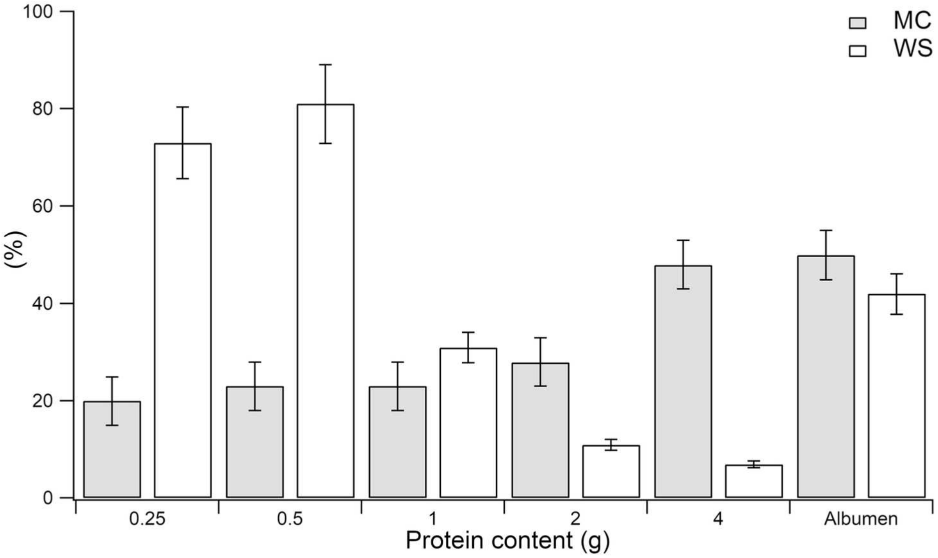


Figure 9 – Moisture content (MC) and water solubility (WS) of bioplastic films tested in the present study and based on different amounts of BSF protein. Comparison with data from literature about albumen based bioplastic. [77]

132x77mm (300 x 300 DPI)

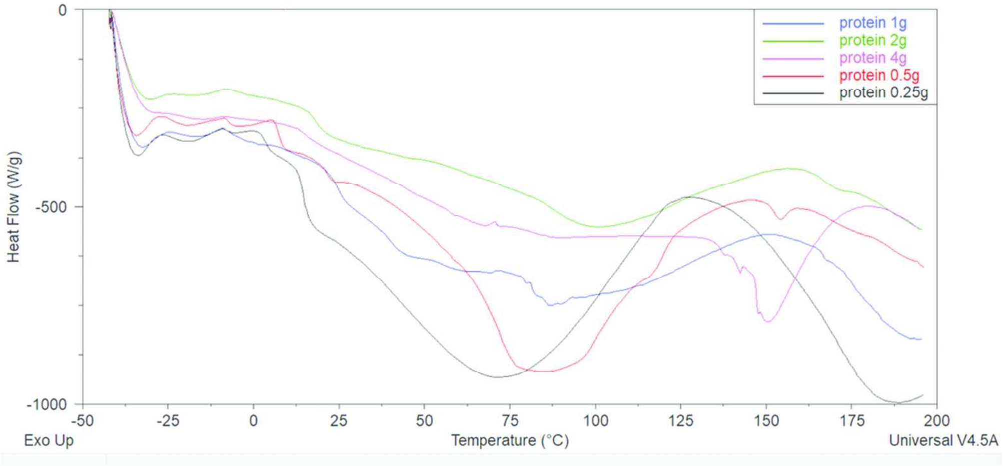


Figure 10 – DSC thermograms of bioplastic films based on different amount of proteins.

99x46mm (300 x 300 DPI)