IOSUD – „DUNĂREA DE JOS" UNIVERSITY DIN GALAȚI

Doctoral School of Fundamental Sciences and Engineering

DOCTORAL THESIS

BIOTECHNOLOGICAL RESEARCH REGARDING THE OBTAINING AND CHARACTERIZATION OF BIOEDIBLE FUNCTIONAL FILMS

PhD Student,

Marian NECULAU

Scientific coordinator,

Prof.dr.ing. Camelia VIZIREANU

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TEZĂ DE DOCTORAT

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(Doctoral thesis summary)

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Key words: bioedible film, biopolymers, *Bacillus subtilis*, yeast extract, onion extract, antioxidant activity.

Scientific objectives and justification for choosing the topic

Foods are naturally susceptible to physical, chemical and microbiological degradation that occurs during their storage and distribution. These changes vary depending on the composition of the food and the parameters of the environment, which can significantly affect the quality of the products. Packaging occupies a central position in food technology (Siracusa et al., 2008). Proper choice of packaging materials and systems is an integral part of the food production process and its design. Lawless et al. (2013) identifies four key roles of packaging: coating / support, protection, ease of use and communication. In fact, each of these roles encompasses several different objectives: technological, engineering and commercial.

The major food manufacturers, together with the manufacturers of ingredients and additives, are continuously investing impressive amounts in research for the development of innovative solutions in order to obtain finished products with a high level of quality and as environmentally friendly as possible. In many economically underdeveloped countries, the World Health Organization has identified that between 30% and 50% of food is wasted due to inadequate conservation and protection, unlike developed economies where the percentage is reduced to 2-3. % (incpen.org, 2016). Here we also find the motto of the Tetra Pack company - “a package must save more than its cost” (tetralaval.com, 2016).

The study entitled "**Biotechnological research regarding the obtaining and characterization of bioedible functional films**" aims to obtain and apply bioedible functional films having as basic ingredients hydrocolloids (easily dispersible polysaccharides in water) such as: sodium alginate, carboxymethyl cellulose, carrageenan and Konjac gum. The films obtained will serve as a support matrix for bioactive ingredients such as yeast extract, onion-based fermented preparate and for a probiotic strain of *Bacillus subtilis* CU1, which will represent the functional part of this film.

The addition of bioactive extracts or probiotic microorganisms selected for the purpose of improving the intrinsic value of food is an increasingly common practice. Researchers in the food industry are focusing on the incorporation of biologically active compounds in bioedible films, especially to extend the shelf life of food (Falguera et al., 2011).

A bio bioedible film or coating has been defined as a continuous thin layer of bioedible material formed on the surface of a food or between various layers of a food or food component. Besides acting as a protective barrier, bio bioedible films can be used as a support for bioactive compounds, increasing the functional properties of the food product due to its benefits (Embuscado et al., 2009). These films are characterized as flexible, thin films obtained from natural polymers of animal or vegetable origin that serve, from a functional point of view, as a support matrix for bioactive substances.

The purpose of bio bioedible films is to ensure a multiple role for the food they serve. At least four defining characteristics of these films can be listed:

- Purpose of food protection as the main protective coating,
- To serve as a matrix for the incorporation of different bioactive substances,
- Superior economic advantages over other competing products on the market,
- Functional purpose of prolonging the validity of the product and improving its main sensory properties.

The research activities within the thesis were carried out with the help, infrastructure and resources of the following institutions:

- Department of Food Science, Food Engineering, Biotechnology and Aquaculture, within the Faculty of Food Science and Engineering, “Dunărea de Jos” University of Galați.
- Food Research Laboratory within the Institute of Food Bioresources in Bucharest.
- Meat pilot plant within the Faculty of Food Science and Engineering, “Dunarea de Jos” University of Galați.
- The laboratory of Kuk Romania SRL, Bucharest.
- Processing plant of Company Sano Vita from Vlădești, Râmnicu Vâlcea.

The structure of the doctoral thesis:

1- The documentary study presents the following information: the current state of knowledge in the field of hydrocolloids used to obtain bioedible functional films; characterization of the main components of bioedible films and exact definition of the notion of bioedible film as well as the history of sodium alginate-based films.

2- The study on the characterization of raw materials and basic materials used to obtain single-layer films will establish a relationship between the main hydrocolloids to improve their characteristics. Each hydrocolloid was carefully analyzed to correlate its properties with the need and optimal dosage of the recipe for a bio bioedible film. Determining the tear strength of the film according to its thickness and determining the maximum applicable diameter depending on the product to be used.

3- The study on obtaining these films and the incorporation of bioactive substances (onion-based fermented preparation, yeast extract and a strain of *Bacillus subtilis* CU1) in order to improve the properties of the film and also to improve the characteristics of the finished product. The experimental study consists of film color and thickness analysis, water vapor permeability analysis, Scanning Electron Microscopy (SEM) analysis, Energy Dispersive X-ray spectroscopy (EDX) and Fourier-transform infrared spectroscopy (FTIR) to study the internal morphology of the film/membrane obtained.

The design of an optimal mixture of hydrocolloids with the addition of active substances to improve the quality of the film, which has the physical parameters at optimal values is an essential objective of the research carried out for the elaboration of the doctoral thesis. In this sense, we applied a systematic approach to process investigation, based on the statistical design of experiments (Design of Experiments - DOE). The use of this technique requires the development of a design model that allows the evaluation of the qualitative parameters of the finished product according to the factors that influence them. In different stages of the experiment (in the statistical analysis of mathematical models) the analysis of variance (Analysis of Variance - ANOVA) and the regression analysis (Regression Analysis) are used as statistical procedures. The experimental design based on Optimal-I Mixture Design was built using the State-Ease Design Expert 11.0 software from CAMO, Norway.

4- Obtaining and applying the bioedible functional film on the surface of complex food matrices of vegetable stick type. In this part of the thesis, microbiological analyzes were performed for two matrices: a vegetable bar packed in collagen / polyamide membrane and the vegetable sausage packed in functional bioedible film. The physical-chemical analysis of both products was performed in parallel with the help of laboratory equipment.

Functional ingredients such as sodium alginate, carrageenan, carboxymethyl cellulose, Konjac gum, which are based on algae-derived polysaccharides, were used to obtain the products

described above. These hydrocolloids have many applications in the food, pharmaceutical, cosmetic, industrial biotechnology and other industries, being used as gelling agents, thickeners or stabilizing and emulsifying agents. Alginate and carrageenan come from two different families of algae: carrageenan is produced by Carrageenanites in the Rhodophyta group, and brown algae alginate in the Phaeophyta family (Draget et al., 2009).

The main objective of this study was to establish a method for obtaining a biofilm based on sodium alginate and other hydrocolloids in which fermented onion-based preparation, yeast extract and pure bacterial culture were added in different proportions from the genus *Bacillus ssp. subtilis*. To achieve the proposed goal, the following specific objectives were pursued:

- Study and characterization of raw materials and basic materials used to obtain single-layer films and to establish a relationship between the main hydrocolloids to improve their characteristics

- Assessment of the possibility of obtaining a bio-bioedible film and incorporating in these polymeric films bioactive substances (onion-based fermented preparation, yeast extract and a strain with special properties of *Bacillus subtilis* CU1 registered under number I-2745 in the national collection of microorganism cultures, CNCM) to improve the properties of the film and also to improve the characteristics of the finished product.

- Studying the functional properties of the bioedible film obtained and evaluating its functionality in the film-food matrix.

The obtained films were evaluated from a microbiological point of view, also following the antioxidant capacity in vitro. The design of an optimal mixture for obtaining an optimized film was made with the help of the DOE-Design of Experiments program, Camo, Norway, the optimization aiming at achieving a balance between performance, quality and cost (Melis, T., 1989).

The doctoral thesis contains 221 pages and is structured in four distinct parts: the “documentary study” presented in 30 pages (10 figures and 4 tables), the “study on the characterization of raw materials and basic materials” which contains 26 pages (15 figures and 1 table), “study on the production of films with incorporated bioactive ingredients” containing 58 pages (27 figures and 22 tables) and “study on the production and application of bio bioedible functional film on the surface of complex food matrices such as vegetable sausage” containing 32 pages (8 figures and 7 tables), conclusions, dissemination of results and related annexes containing 58 pages.

The bibliography of this paper was passed at the end of each chapter and totals a number of 16 pages with most of the references cited after 2010 to reveal the news in this field.

II. Physical, chemical and rheological characterization of hydrocolloids used to obtain bioedible functional films

2.1. Introduction

This chapter includes the study and the characterization of raw materials and basic materials used to obtain single-layer films and the relationship established between the main hydrocolloids to improve their characteristics. Each hydrocolloid was carefully analyzed to correlate its properties with the need and optimal dosage of the recipe of a bioedible film. Determining the shear strength of the film according to its thickness and determining the maximum applicable diameter depending on the product to be used.

2.2. Materials, equipment and methods of analysis used in the experiments

Materials

In the present study, determinations were made regarding the rheological behavior of four hydrocolloid solutions as follows:
P1 - CMC-carboxymethyl-cellulose, trade name product: 7H9, manufacturer Ashland, USA.
P2 - Sodium alginate, trade name: Ferwo Alginate F400, manufacturer Caldic Ingredients, Rotterdam, Netherlands.
P3 - Refined Kappa-carrageenan, product trade name: BLK 1120, manufacturer BLG-Brilliant Gum, Shanghai, China.
P4 - Konjac mannan, product trade name: MRA, manufacturer Food Ingredients & Solutions, Netherlands.

Equipment: pH meter, Metrohm type, Switzerland; analytical balance, type Kern ADB 200-4, Germany; Phillips vertical mixer, China; calcination furnace, Proteherm PAF 110/6, Germany; centrifuge Funke Gerber, Germany; 100 mL graduated cylinder, Isolab, DIN / ISO class B, Germany; glass funnel, diameter 55mm, Isolab, Germany; 400 mL Berzelius glasses, Isolab, Germany; Petri dishes, diameter 90 cm, Isolab, Germany; granular dryer, Marienfeld, Germany; 50 mL crucibles, tall, Isolab, Germany; gas nozzle; rheometer AR 2000, TA Instruments, USA; Peltier plate as an accessory of the AR 2000 Rheometer; viscometrical cup with interchangeable holes from TQC Instruments, The Netherlands; viscometrical cup hole Type 4, DIN Standard; viscometrical cup support tripod, TQC, Netherlands; electronic stopwatch, Isolab, Germany.

The investigations aimed:
- physical, chemical and sensory characterization of biopolymers
- Determining the rheological parameters of the analyzed hydrocolloids
- Influence of concentration on the viscosity of hydrocolloid solutions

2.3. Results and discussions

a. Physical, chemical and sensory characterization of biopolymers

In table 2.1. the physical, chemical values of the analyzed samples are indicated. The samples are presented in fine powder form with small granulation, below the value of 90 µm. Their color varies from dark white to brown, depending on the raw material from which they are obtained.

For samples P3 and P4, the shades of dark white and brown are specific to the algae powders that form the basis of these raw materials. In the case of pH, there is a high value of 9.15 units for P4, due to the method of obtaining the hydrocolloid.

Table 2.1. The values of the physical, chemical and organoleptic characteristics of the analyzed samples

Parameter	CMC	Alginate	Carrageenan	Konjac
Appearance	Fine powder	Fine powder	Fine powder	Powder
Color	Dark white	white	Dark white	brown
pH	8,02±0,04	7,61±0,06	7,40±0,07	9,15±0,07
Moisture (%)	4,97±0,01	7,07±0,02	6,75±0,01	8,51±0,01
Volumetric density (g/ml)	0,58±0,02	0,62±0,05	0,62±0,02	0,62±0,01
Pressed density (g/ml)	0,78±0,03	0,84±0,02	0,94±0,06	0,95±0,07
Carr Index (%)	24,70±0,09	26,25±0,13	33,33±0,12	34,78±0,16
Hausner Ratio	1,32±0,12	1,35±0,15	1,50±0,17	1,53±0,14
Water holding capacity (%)	12,46±0,12	12,17±0,15	13,73±0,16	12,00±0,11
Viscosity at 1% (cSt)	26,67±1,25	43,67±0.94	16,67±1,25	682±2,45
Ash (%)	0,95±0,01	0,65±0,02	0,66±0,01	0,22±0,03

All values represent Means ± Standard deviation, n=3, ($p<0,05$).

For samples P2 and P3 similar pH values were recorded, values close to neutral pH. Similar results were reported by Belalia et al. (2014) for sodium alginate. For each hydrocolloid, the pH values were significantly different ($p <0.05$) per line with large pH variations from 7,40 to 9,15 units. The moisture of the samples differs depending on the method applied by the manufacturer to dry the hydrocolloids analyzed. The highest moisture value of 8,51% is recorded at P4, while the sample with the lowest moisture has the P1 sample with a value of 4,97%. In the case of the analysis of water holding capacity, similar values were obtained for samples P1, P2 and P4 of approximately 12%. A higher value was marked by P3 (about 13,73%) due to the specific structure of carrageenan and how to obtain and standardize it with the help of potassium salts (Bixler, Porse, 2011).

The viscosity values of samples P1-P4 are within the normal parameters for these hydrocolloids for a solution concentration of 1%, similar values being noted by Laaman (2011), although for the determination of the viscosity of hydrocolloids there were differences in the values obtained due to various procedures and equipment used. The viscosity of the samples analyzed as a 1% solution recorded close values for P1, P2 and P3. In the case of sample P4, the viscosity reached a value of 682 cSt due to the ability of Konjac gum to form gels.

b. Determining the rheological parameters of the analyzed hydrocolloids

Figure 2.2. and 2.3. shows the flow curves of the dispersions and gels analyzed for the sodium alginate (AL) and carboxymethylcellulose (CMC) samples. In figure 2.2. it is observed that, from a

rheological point of view, the alginate sample showed a different behavior compared to the CMC sample by different values of the viscosity of the gels. In this case, at low shear rates up to 0,5 s^{-1}, the alginate gel showed a dilatancy behavior (the viscosity increased from 0,7 Pa × s to 0,9 Pa × s).

Moreover, the viscosity value could be recovered on the return curve only up to a speed of 2 s^{-1}, after which, at very low shear rates, the viscosity of the alginate gel decreased constantly, to a final value of about 0,45 Pa × s, much lower than that recorded at high shear rates, thus emphasizing the dilatancy nature of the gel. In general, sodium alginate solutions tend to be very viscous. The behavior of the alginate gel at high shear rates ($\gamma > 10^2$ s^{-1}) makes it a suitable material for bioedible films due to the low viscosity of the material after this point (η = 0,55 Pa × s).

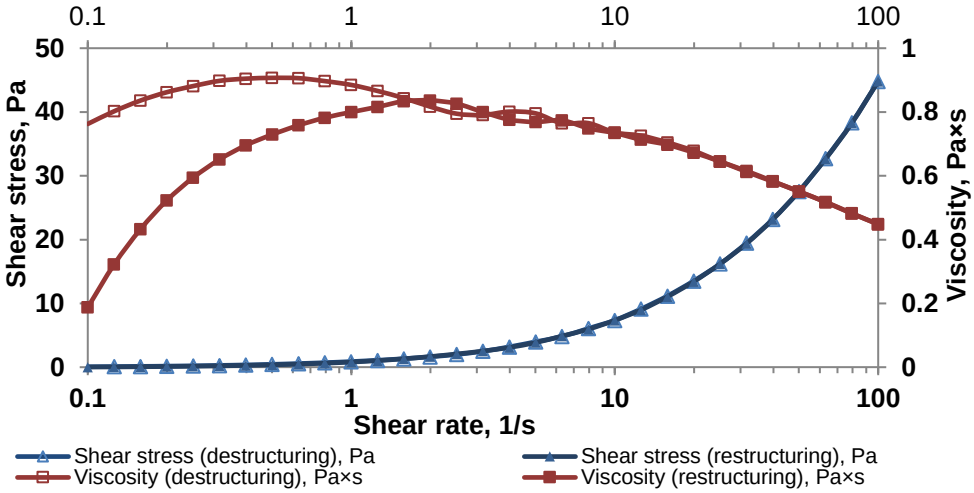


Figure 2.2. Variation of rheological parameters (η and γ) for alginate samples. Flow curves for the formed gel.

Figure 2.3. presents the rheological profiles of the CMC samples in the form of flow curves in semilogarithmic representation for the viscosity expressed in Pa × s and the shear rate expressed in s^{-1}. The results indicated the pseudoplastic character of the gel (decrease in viscosity with increasing shear rate), but the viscosity value could be recovered on the restructuring curve of the CMC sample up to 0,1 s^{-1} (at very low shear rates) thus exceeding the initial viscosity value of 55,83 Pa × s. It was also possible to observe the phenomenon of hysteresis, but whose area was relatively small (between the values of 17,69 Pa × s and 30,84 Pa × s on the viscosity restructuring curve). Similar results attesting to the CMC's ability to be a good crosslinking agent in obtaining bioedible films were also noted by Mu et al. (2012).

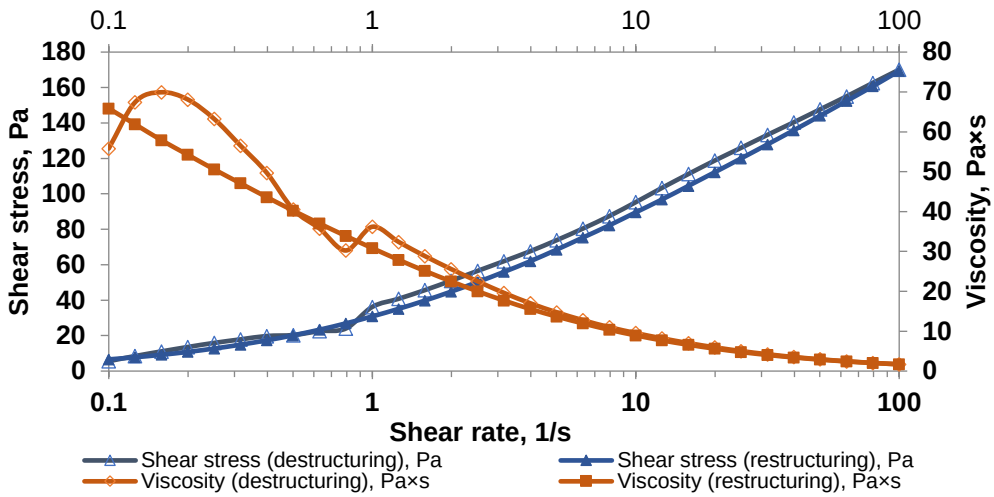


Figure 2.3. Variation of rheological parameters (η and γ) for CMC samples. Flow curves for the formed gel.

The viscosity of the CMC sample reaches the value 0 under conditions of maximum shear stress at an estimated value of 170 Pa. The analyzed sample is a suitable ingredient for obtaining bioedible films under the conditions of applying maximum torque.

In figures 2.4., 2.5., 2.6. and 2.7.the rheological profiles of alginate, CMC, carrageenan and Konjac samples were represented by the gel frequency scan test.

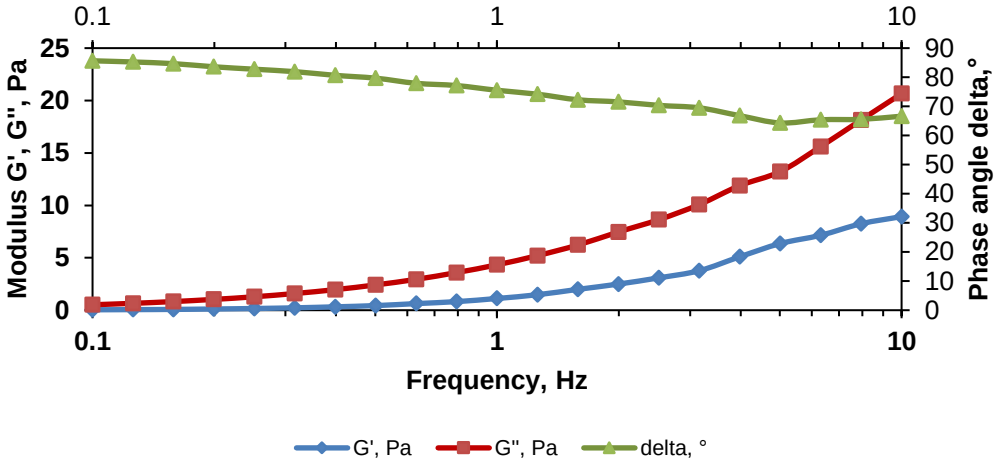


Figure 2.4. Variation of rheological parameters (G', G'' and delta) for alginate samples. Gel frequency scan test.

In figure 2.4. for the frequency scanning test, a behavior could be observed were the values of the viscosity modulus G'' were higher than the values of the elastic modulus G', providing a viscous character for the material. In the first part of the frequency range, up to the value of 1 Hz, both the elastic modulus and the viscous modulus constantly evolve with small variations of the values framed at 1,1 Pa and 4,3 Pa respectively, following that after this frequency, until the end of the test, to record maximum values of 8,9 Pa and 20 Pa respectively for the two modules. The higher the frequency, Hz, on the analysis field, the higher the G' and G'' modules for the alginate sample, which can be correlated with an increase in the number of final free alginate chains due to dimerization (chemical reaction between two random free radicals) in the heterogeneous network (Palmer et al., 2014).

Alginate samples show a viscoelastic behavior in this case. The viscoelastic nature of the three-dimensional networks of alginate as well as of other polysaccharide systems can be explored by dynamic measurements that allow the determination of G' and G" modules.

In the case of the frequency scanning test, the evolution of the viscoelasticity modules G' and G" was observed. In Figure 2.5. as the frequency increased, the CMC gel became more elastic, having a G' with higher values, the viscous component of the CMC gel being lower than the elastic modulus, growing at a much slower rate. However, for low oscillation amplitude frequencies (imposed at 1%), from 0,1 to 0,2 oscillations per second, the viscous component had higher values than the elastic component, which can be translated by the fact that under quasi-static conditions, the CMC gel is more viscous than elastic, behaving like a fluid (flowing).

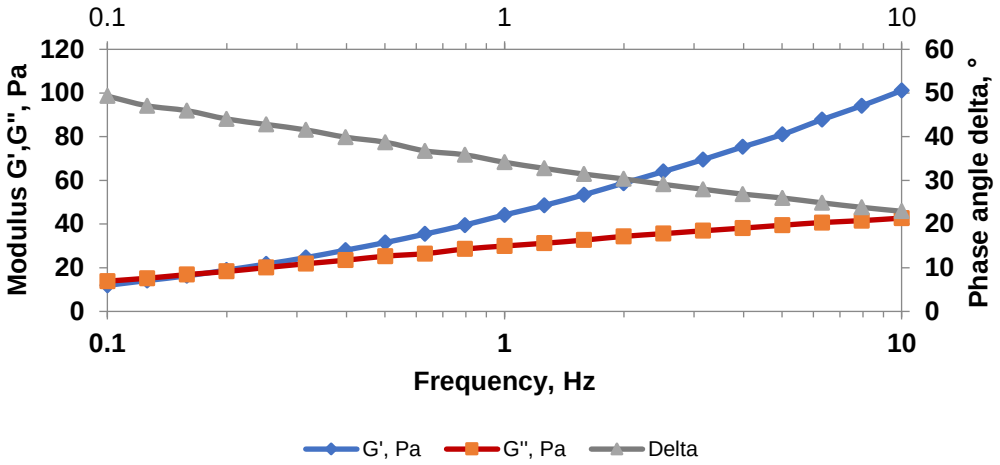


Figure 2.5. Variation of rheological parameters (G', G" and delta) for CMC samples. Gel frequency scan test.

Delta, representing the phase angle of the oscillations, decreased from 49° to 23°. Having the viscos/elastic delimitation around 45°, we can also observe through the evolution of this parameter the transition from a predominantly viscous behavior to a predominantly elastic one. We can mark the frequency of approximately 0,2 Hz as the inversion point between G' and G''. Similar results were obtained in the case of PVP-CMC hydrogels in which the elastic modulus G' registers much higher values than the viscous modulus G'' (Niladri et al., 2011).

In figure 2.6. the rheological profile for Kappa-carrageenan gel (KC) is presented, which showed a predominantly elastic character throughout the frequency scanning test with the elastic component G' higher than the viscous component G".

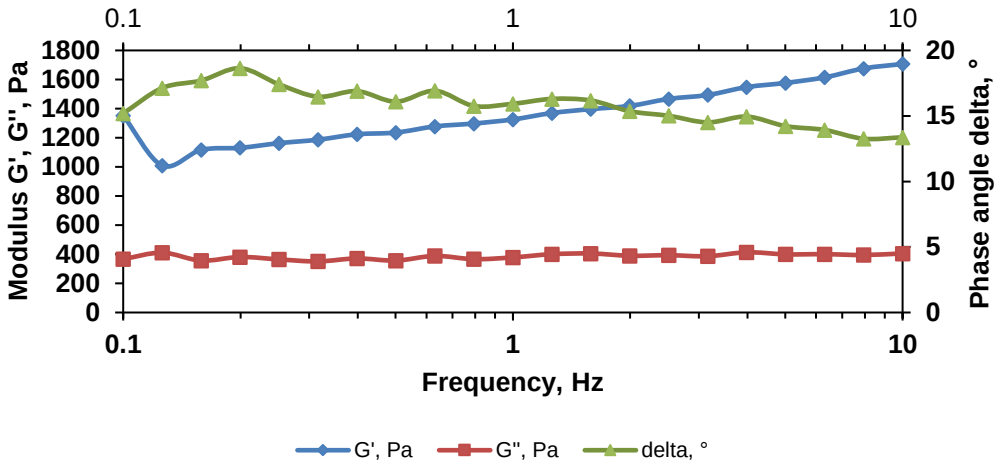


Figure 2.6. Variation of rheological parameters (G', G" and delta) for kappa-carrageenan samples. Gel frequency scan test.

The large difference between the values of the two modules confirms that, in the case of the frequency scan test, the KC gel behaves like an authentic solid. True gels are formed by enough junction areas in KC solutions in the presence of sufficient salts (Brenner et al., 2013; Brenner et al., 2015).

It can be noticed that, at low values of the frequency of 0,13 Hz, the viscosity modulus G" registered a slight increase of the value, and the elastic modulus G' registers a slight decrease of the value, but these changes are small and do not significantly influence the behavior of carrageenan samples during the test. The phase angle of the oscillations, delta, registered a decrease along the entire length of the analyzed range from 15,19° to 13,37°. In contrast, the elastic component G' increases with increasing frequency over the entire length of the analyzed range from 1352 Pa to 1707 Pa. The viscous component G" marks a linear path along the entire length of the analyzed interval, with a slight increase at the end of the range, registering values from 367 Pa to 405 Pa. Similar results regarding the evolution of viscoelasticity modules for KC gels, as well as for Konjac (KG) gum gels, were also obtained by He Xue et. al. (2012).

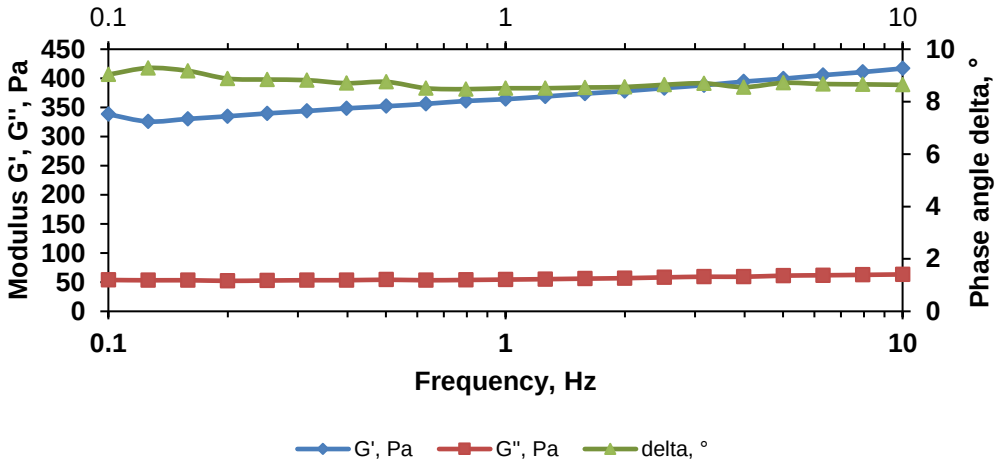


Figure 2.7. Variation of rheological parameters (G', G" and delta) for Konjac gum samples. Frequency scan test.

In figure 2.7. the rheological behavior of the Konjac gum suspension (KG) can be observed as a result of the oscillation frequency scan. The behavior of the KG gel remained similar to that of the KC gel. We also identify for the KG gel a predominantly elastic character, in which G' is greater than G" throughout the frequency (0,1-10 Hz).

At the same time, both viscoelastic modules register a slight increase on the whole analyzed field, with the increase of the frequency values. Using the strain scan test, in figures 2.8., 2.9., 2.10. and 2.11., the linearity of the material for alginate gels, CMC, carrageenan and Konjac was analyzed.

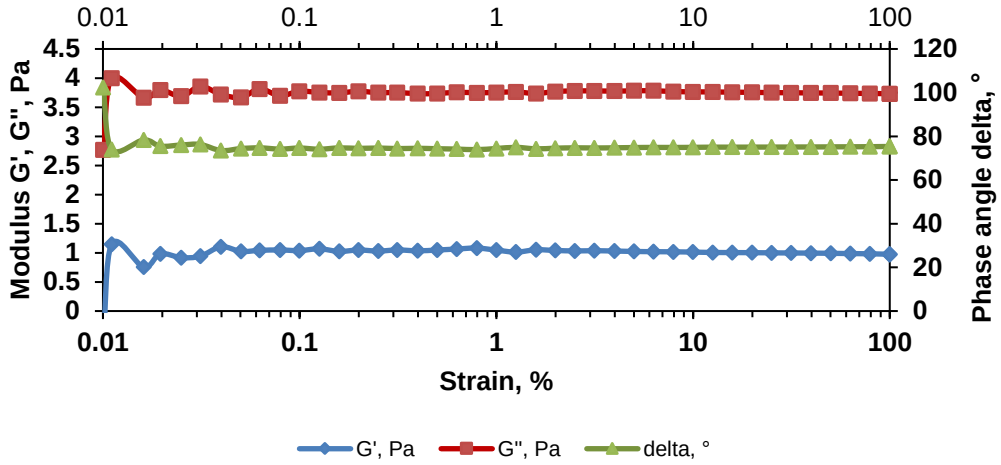


Figure 2.8. Variation of rheological parameters (G', G" and delta) for alginate samples. Strain scan test for alginate gel.

Unlike the CMC gel (figure 2.9.), where the limit of the viscoelasticity range could be registered, in the case of the alginate gel the values of the viscoelasticity parameters remained constant throughout the test, the matrix remaining stable even at 100% strain. However, unlike CMC, the alginate gel had a viscous character, with G" values marking an average of 3,75 Pa prevailing over G' values with an average of less than 1 Pa, and the delta angle values being very close of those of a fluid. Similar results for the strain scan test of alginate gel samples were obtained by Belalia et al. (2014) with G" > G'. The combination of a high molecular weight polymer and a surfactant forms a high viscosity complex resistant to large strains.

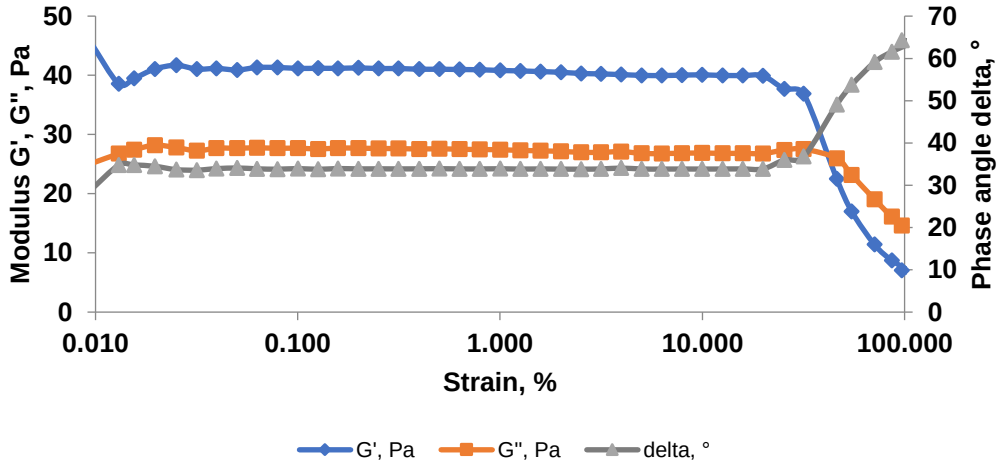


Figure 2.9. Variation of rheological parameters (G', G" and delta) for CMC samples. Strain scan test for CMC gel.

In Figure 2.9., for the strain scan test, we can observe that up to a strain of 25%, the elastic modulus G', the viscous modulus G" and the delta angle remained approximately constant as evolution, the material behaving like a solid with G '> G". After the 25% threshold of strain, the exit from the viscoelasticity range was observed, being also registered a phase change at a deformation of approximately 46%, when the gel started to flow, the viscous component predominating over the elastic component. Following the strain scan test, it was possible to establish the critical strain of the viscoelasticity domain, and similar results regarding the evolution of the two modules were obtained by (Stephen et al., 2016), where at low strain the value of the elasticity mode G' is greater than the value of the viscosity mode G" for various CMC types.

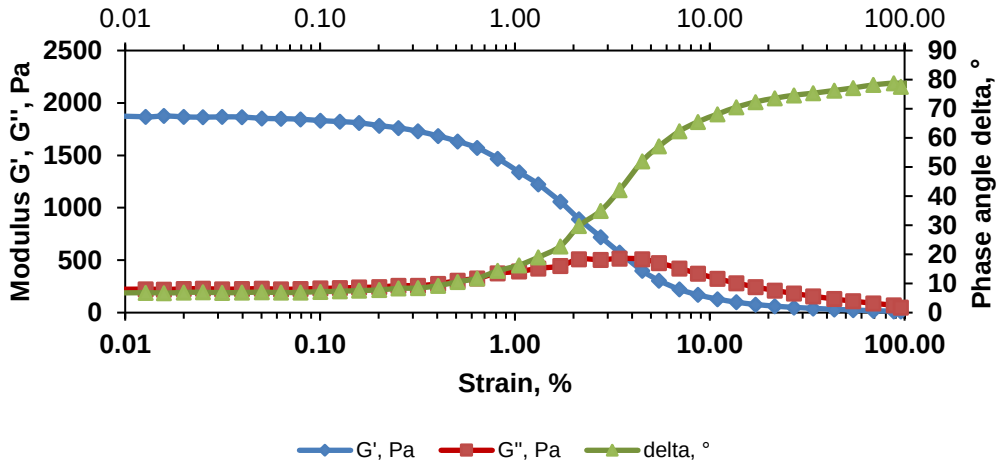


Figure 2.10. Variation of rheological parameters (G', G" and delta) for carrageenan samples. Strain scan test for carrageenan gel.

Figure 2.10. illustrates the rheological profile of the carrageenan sample. Carrageenan gel did not have a stable structure, the viscoelasticity range can be recorded only up to strains of maximum 1%, after which, at amplitudes of oscillations of 3,5% the phases were reversed, the viscous component prevailing over the elastic one. The material becomes fluid. The strength of the colloidal forces is indicated by tan δ = (G"/ G'). A tan δ with a value of less than 1 suggests that the particles are strongly associated due to colloidal forces and thus sedimentation may occur.

A value greater than 1 for tan δ suggests that the particles are strongly dissociated. The critical strain for electrostatically stabilized systems are around 0,01% to 0,5%, and for sterically stabilized systems between 1% and 5% as in this case. The results for the carrageenan sample are somewhat similar to the CMC sample with the difference that the phase change occurs much faster for the carrageenan sample at a strain of 3,44%, unlike the CMC sample where the phase change occurs at a strain of 46%.

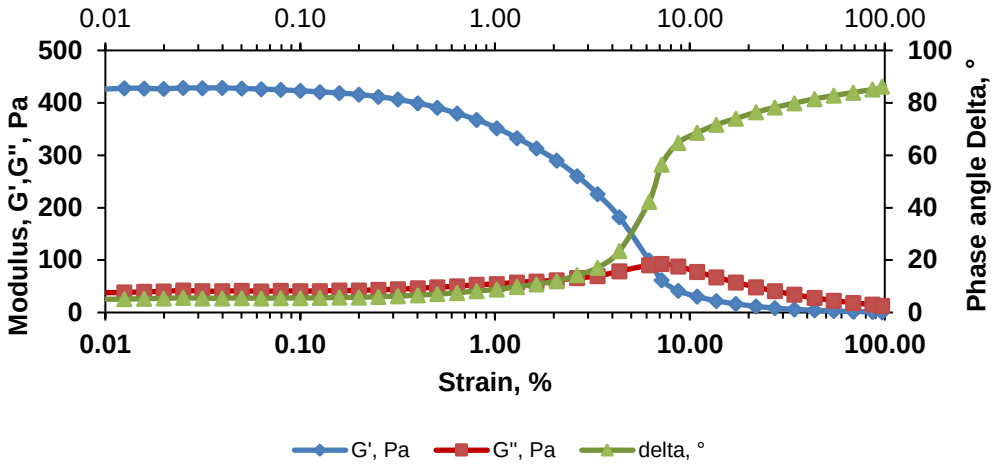


Figure 2.11. Variation of rheological parameters (G', G" and delta) for Konjac mannan samples. Strain scan test for Konjac gum gel.

In Figure 2.11, the behavior of the Konjac gel during the oscillation amplitude scan test was relatively identical to that of the carrageenan gel. The material was stable to around 1,5% strain after which it began to flow. Similar results were recorded by (He Xue et al., 2012), which by combining hydrolyzed Konjac glucomannan with carrageenan showed an increased strength of the gel formed.

In this case, G' is larger than G" on a significant surface of the deformation range.

c. The influence of concentration on the viscosity for hydrocolloid solutions

The evolution of the viscosity for the alginate and CMC samples was illustrated in Figure 2.12., 2.13. and a gradual increase is noticed with the increase of the hydrocolloid concentration in the solution in a certain time interval. In the case of alginate solutions, the viscosity gradually increased from 27 cSt for a solution concentration of 0,5%, to a viscosity value of 208 cSt when the solution has a maximum concentration of 1,5%. The graph shows a linear increase throughout the range. In the case of CMC solutions, the viscosity increased from 10 cSt for a solution concentration of 0,5% and reached a maximum of 42 cSt for a maximum concentration of 1,5%.

The analyzed alginate samples recorded much higher viscosity values as opposed to CMC samples, both at low concentrations of 0,5% and at maximum concentrations of 1,5%. In Figures 2.12 and 2.13, the time (expressed in seconds) indicates a higher value for alginate solutions than for CMC due to the higher viscosity of the alginate that flowed through the viscometrical cup over a much longer period of time.

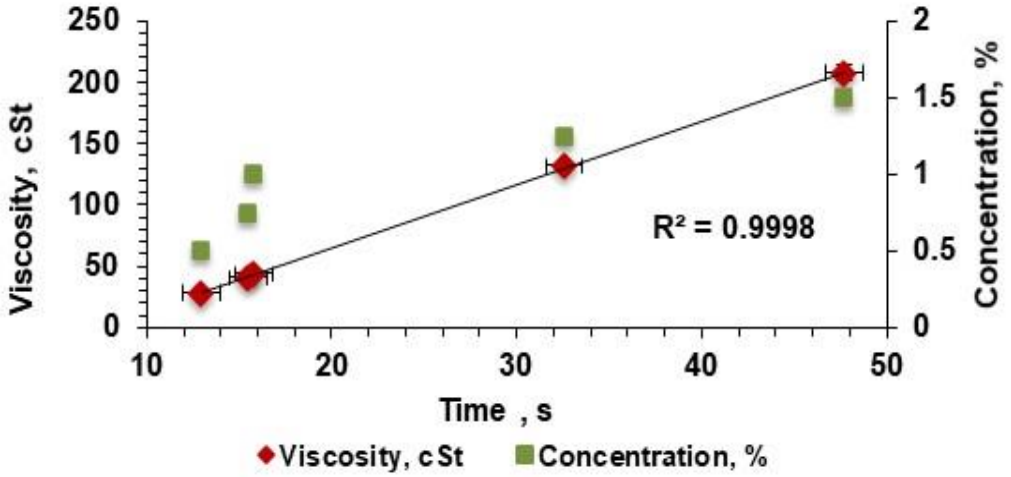


Figure 2.12. Evolution of the viscosity for alginate samples as a function of concentration and time.

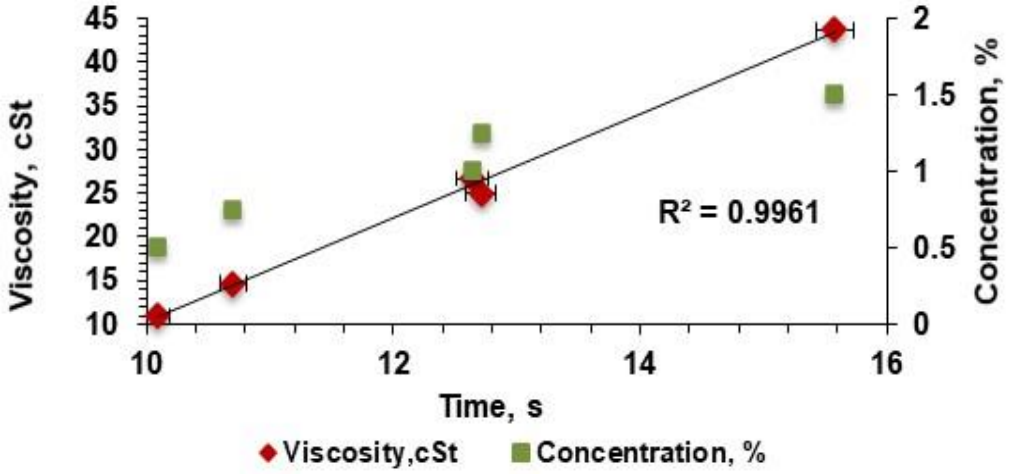


Figure 2.13. Evolution of the viscosity for CMC samples as a function of concentration and time.

This property of CMC is due to the internal structure of the polysaccharide, a fact highlighted in the previously reported viscoelasticity experiments. In figure 2.12. and in figure 2.13. the statistical analysis indicates probability values with $p < 0.05$ for the viscosities of the two hydrocolloids validating the variations between and within the analyzed groups. The carrageenan and Konjac samples were illustrated in Figure 2.14., 2.15. and indicated a gradual increase in viscosity, but at different values of alginate and CMC solutions.

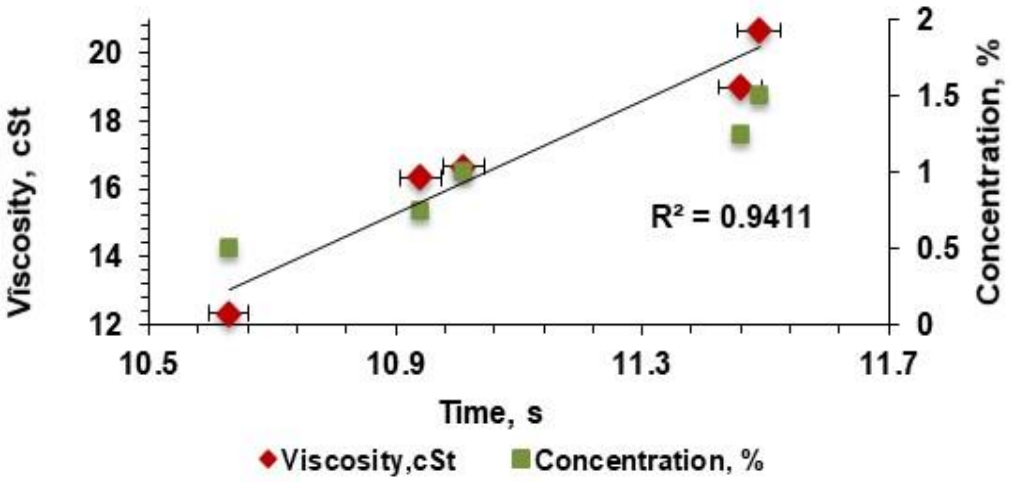


Figure 2.14. Evolution of the viscosity for carrageenan samples as a function of concentration and time.

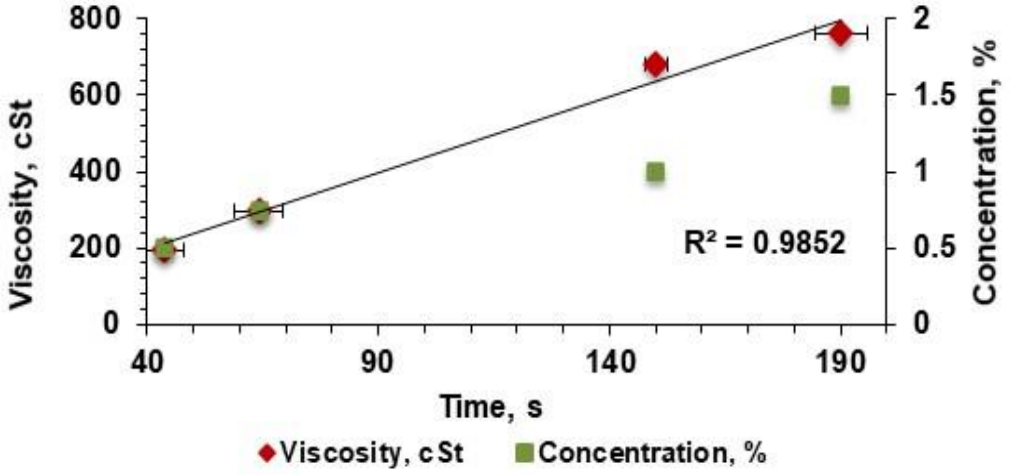


Figure 2.15. Evolution of the viscosity for Konjac samples as a function of concentration and time.

The viscosity of carrageenan solutions increased from the value of 12 cSt from a concentration of 0,5%, to a value of 18 cSt and a concentration of the solution of 1,5%. In the case of Konjac solutions there was an unexpected increase in viscosity, namely from a value of 190 cSt and a concentration of 0,5% to a value of 682 cSt and a concentration of only 1,0%, the determinations of the viscosity of Konjac solutions at concentrations higher than 1.5% being practically impossible due to the very viscous character of the analyzed material. It could be noticed that the Konjac solutions were very viscous at low concentrations as opposed to the carrageenan solutions. Another indicator of the higher viscosity of Konjac was the time when the solution managed to flow out of the viscometrical cup, reaching a value of 150s and a solution concentration of 1,0% and a maximum viscosity value. An increase in viscosity over time (hours and days) for samples of Konjac flour and Konjac gum was reported by (W. Xu et al., 2014), but in which the sample concentration was limited to a maximum of 1%.

As an indicative classification, it could be noted that the Konjac and alginate solutions were the most viscous at both low concentrations of 0,5% and higher concentrations of 1,5%. These two materials were followed in the order of viscosity by the solutions of CMC and carrageenan,

respectively, whose values obtained are much lower. In both figures 2.14. and 2.15., $p < 0.05$ for the carrageenan and Konjac gum viscosity values.

2.4. Partial conclusions

- The flow curves of samples P1 and P2 (alginate and CMC) indicated specific values for the two hydrocolloids. In the case of CMC gel the shear rate was inversely proportional to the viscosity, meaning the higher the shear, the lower the viscosity of the gel, unlike the alginate gel which at low shear rates showed an increase in viscosity and at high shear rates showed a decrease in it.
- In the case of the frequency scanning test, the CMC gel recorded at low amplitudes of the oscillations a predominantly viscous behavior G"> G', and at amplitudes of oscillations above values of 0,2 Hz G'> G" was recorded, thus indicating a predominantly elastic character of the product. In the case of the alginate gel, throughout the test G”> G’, thus marking a predominantly viscous character. A similar behavior was recorded in carrageenan and Konjac gels, in which throughout the test G '> G ", marking samples with an elastic character.
- In the case of the strain scan test, the following conclusions were noted: for the CMC gel at strain <25%, the components G', G” and delta were constant as evolution. At strain > 25% the CMC gel started to flow, at strain of 46%, G"> G'. In the case of the alginate gel throughout the test, the gel had a fluid behavior (flow) and G” > G’. Similar behaviors were reported in carrageenan and Konjac gels up to strain of 3,5%, G'> G" (solid behavior), and to strain over 3,5%, G"> G' (fluid behavior).
- The data obtained by relating the viscosity to the solution concentration and time for alginate and CMC indicated a much higher viscosity value for alginate solutions as opposed to CMC solutions over the whole range of concentration variation for the two hydrocolloids.
- In the case of the viscosity of carrageenan and Konjac solutions, the situation was the opposite of the viscosity of alginate and CMC solutions. The viscosity of Konjac solutions was higher than that of carrageenan solutions even at very low concentrations of Konjac gum. This was possible due to the structure of the Konjac gum which had a favorable spatial orientation to the addition of water molecules and, implicitly, to the formation of a high viscosity even at very low concentrations of the solution.
- The individual analysis of the P1-P4 samples may indicate certain correlations and synergies between them. In the case of the Konjac and CMC samples, the viscous character predominated, unlike the alginate and carrageenan samples, in which the elastic character predominated. The balance between the two elements that can be essential for obtaining quality bioedible films.

III. Study of bioedible functional films based on sodium alginate

3.1. Introduction

This chapter includes the study on the production of films and the incorporation of bioactive ingredients (onion-based fermented preparation, yeast extract and a probiotic strain of *B. subtilis* CU1) in order to improve the properties of the film and also to improve the characteristics of the finished product. The experimental study consists of film color and thickness analyzes, water vapor permeability analysis, Scanning Electron Microscopy (SEM) analysis, Energy Dispersive X-ray spectroscopy (EDX) and Fourier-transform infrared spectroscopy (FTIR) to study the internal morphology of the film coating obtained.

3.2. Materials, methods of analysis and equipment used in experiments

The following materials with the corresponding coding were used in the experiments: [CMC] - sodium carboxymethyl-cellulose, trade name product: 7H9, manufacturer Ashland, USA; [AL] - Sodium alginate, product trade name: Ferwo Alginate F400, manufacturer Caldic Ingredients, Rotterdam, The Netherlands; [RC] - Refined Carrageenan, product trade name: BLK 1120, manufacturer BLG-Brilliant Gum, Shanghai, China; [KG] - Konjac mannan, product trade name: MRA, manufacturer Food Ingredients & Solutions, Netherlands; Bacillus subtilis CU1, trade name Probisis *B. subtilis* CU1, manufacturer LeSaffre Human Care, France and registered with no. CNCM I-2745; Selected culture medium Luria Bertani Agar, Miller, producer Titan Biotech Ltd., Rajasthan, India; [ED] - Yeast extract, trade name Springer 4101/0, manufacturer Bio Springer Company, Maison Allfort, France; [PFC] - Onion-based fermented preparation, trade name SLR 100, manufacturer Fi & S, Netherlands; Glycerol and Oleic Acid - used as plasticizers, manufacturer Sigma Aldrich, Germany; CaCl2 used as a crosslinking agent was supplied by Sigma Aldrich Company; 2,2'-Diphenyl-1-picrylhydrazyl radical (DPPH) products sold / traded by Sigma Chemicals Co.

The investigations aimed:
- Rheological analysis of sodium alginate films
- The antioxidant capacity of the bioactive ingredients which are part of the film
- Microbiological analysis of sodium alginate-based films
- Permeability of sodium alginate films
- EDS and SEM analysis
- FTIR analysis
- Experimental design

3.2.2. Methods of analysis and the equipment used in experiments

3.2.2.1. Rheology of alginate-based films

The rheological characterization of alginate-based gels and films was performed using the AR 2000EX Rheometer from TA Instruments using a plate with conical geometry, with a conical angle of 1° and a diameter of 50 mm. In table 3.1. the basis of the hydrocolloid mixture is illustrated.

Table 3.1. The mixture of hydrocolloids used to obtain the base of bioedible films.

No.	Hydrocolloid UM	P1 g	P2 g	P3 g	P4 g	P5 g	P6 g	P7 g
	Film mixture	AL	AL+RC	AL+KG	AL+CMC	AL+RC+KG	AL+RC+ CMC	AL+KG+ CMC
1	Alginate	6	5	5	5	5	5	5
2	Carrageenan	-	1	-	-	0.5	0.5	-
3	Konjac Gum	-	-	1	-	0.5	-	0.5
4	CMC	-	-	-	1	-	0.5	0.5
5	Distilled water	400	400	400	400	400	400	400
6	**Total**	**406**	**406**	**406**	**406**	**406**	**406**	**406**

3.2.2.3. Microbiological analysis of alginate-based films

a. Viability of *Bacillus subtilis* CU1 strain in the mix of bioactive ingredients

The experiment started from the premise that the pure strain of *Bacillus subtilis* CU1 contains according to the data sheet and the certificate of analysis a concentration of microorganisms > 10^{11} cfu / g (viable spores). In table 3.2. the system for preparing the bioactive ingredients for 100 ml of solution is illustrated.

Table 3.2. Bioactive ingredient preparation system (quantities per 100 ml solution**)**

Sample	*B. subtilis* CU1 (g)	YE(g)	OFP(g)
M	0,25g	-	-
P1	0,25g	1,0g	-
P2	0,25g	-	1,0g
P3	0,25g	1,0g	1,0g

b. Viability of *Bacillus subtilis* CU1 strain in alginate-based films with incorporated bioactive ingredients

Recipes for active alginate sodium-based films have been illustrated in Table 3.3. The hydrocolloid mixture was chosen according to the rheological compatibility and breaking strength of the alginate-based films analyzed in the previous subchapter.

Table 3.3. Mixture of bioactive ingredients of alginate-based films per 100 ml solution

Film recipe no. 1	*B. subtilis* CU1 *(g)*	YE (g)	OFP (g)
1A: AL + KG + CMC	0.500g	0.500g	0.500g
1B: AL + KG + CMC	0.625g	1.000g	1.000g
1C: AL + KG + CMC	0.750g	1.500g	1.500g
Film recipe no. 2			
2A:AL+ KG+ CMC+ RC	0.500g	0.500g	0.500g
2B:AL+ KG+ CMC+ RC	0.625g	1.000g	1.000g
2C:AL+ KG+ CMC+ RC	0.750g	1.500g	1.500g

The experiment followed the bellow dosages:

AL [1,125g] + KG [0,1875g] + CMC [0,1875g] = 1,5g **(3.2)**

AL [1,0275g] + KG [0,1575g] + CMC [0,1575g] + RC [0,1575g] = 1,5g **(3.3)**

3.2.2.6. Permeability of alginate-based films

The permeability of sodium alginate films was determined gravimetrically according to ASTM E96-93. Table 3.6. presents the method for the determination of WVTR and WVP for the control sample (M) and for the sample (P1) which has variations described by the sample code: a, b, c and d. The differences refer to the ambient temperature at which the samples are left for 24 hours and at the relative humidity inside and outside the analyzed samples. Two methods describe this analysis: The method with siccative "water vapor transfer is performed from the external environment inside the cup" and "Distilled water method", water vapors are transferred from the inside to the outside, the inside of the cup is filled with distilled water.

Table 3.6. Samples M and P1 for measuring the water vapor permeability of bioedible films (adapted according to ASTM E96).

Method	Sample	Code	t, ℃	RH%, glass g mm/kPa day m^2	RH%, external g mm/kPa day m^2
Method with siccative	M	a	8	0	55%
Method with siccative	M	b	22	0	50%
Method with D water	M	c	8	100	55%
Method with D water	M	d	22	100	50%
Method with siccative	P1	a	8	0	55%
Method with siccative	P1	b	22	0	50%
Method with D water	P1	c	8	100	55%
Method with D water	P1	d	22	100	50%

The permeability and transmission rate of water vapor was carried out as follows:

Table 3.7. Amounts of plasticizer (glycerol and / or oleic acid) added to the recipe of sodium alginate-based films.

Sample	Glycerol, (g)	Oleic acid, (g)
M	0	0
P1	0,675	0
P2	0,900	0
P3	1,125	0
P4	0,675	0,675
P5	0,900	0,900
P6	1,125	1,125

3.3. Results and discussions

3.3.1. Rheology of sodium alginate films

In figure 3.3. the mechanical strength of seven films (P1-P7) is represented to determine which of the proposed samples has an increased strength and can subsequently represent a basis for the matrix of active components. All samples analyzed were based on sodium alginate as the major hydrocolloid. The rheological profiles of the samples (AL + CMC) and (AL + KG + CMC) highlighted the linear and uniform characteristics of an optimal film in terms of mechanical strength.

In this case the elastic modulus G' was limited in the range 4-5 × 10^6 Pa and was stable along the entire length of the analysis range.

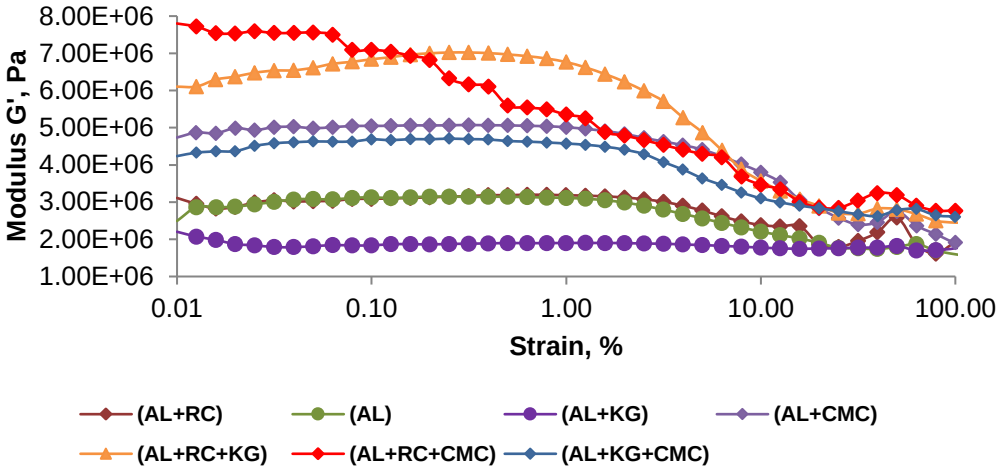


Figure 3.3. The mechanical strength and structural integrity of the G' modulus in sodium alginate-based films.

The samples (AL + RC + KG) and (AL + RC + CMC) showed a more obvious elastic behavior, due to the high values of the G' modulus at low amplitudes of the oscillations than the previously analyzed samples. This may be due to a stronger structure of the hydrocolloid mixture, with a specific solid behavior, but which is not stable along the entire range of the analysis. This instability of the hydrocolloid mix does not recommend the two samples for increased mechanical strength. In the case of samples (AL), (AL + KG) and (AL + RC) the recorded mechanical strength showed much lower values than in the case of the first two analyzed samples.

In figure 3.4. the G" (plastic) modulus of sodium alginate-based films was illustrated. Usually this characteristic is correlated with the G' (elastic) modulus that characterizes the film. In this case there was a significant increase in modulus G" when the strain reached 50% for samples (AL + RC), (AL + RC + CMC) and (AL + CMC) respectively. At the opposite pole, the samples that registered a certain constancy throughout the strain were represented by (AL), (AL + KG + CMC) and (AL + RC + KG) respectively.

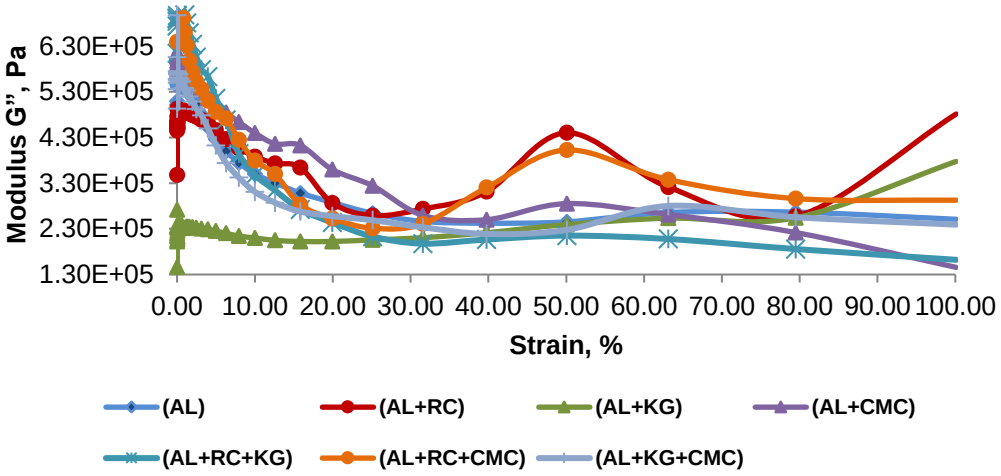


Figure 3.4. The mechanical strength and structural integrity of the G" module in sodium alginate films.

In both figures 3.3. and 3.4. samples P3 and P7 were highlighted due to both viscoelastic modules (G' and G") which indicated a linear and relatively constant behavior throughout the analysis field. This was also highlighted in terms of the mechanical strength of the two samples, noted in Table 3.10.

Table 3.10. Mechanical strength of alginate-based films.

No.	Sample	Hydrocolloid mixture	Gel ph	Film breaking point, (MPa)
1	P1	AL	7,25±0,03	50,09±0,01
2	P2	AL+RC	6,91±0,06	28,39±3,26
3	P3	AL+KG	7,1±0,01	Not breaking
4	P4	AL+CMC	7,49±0,01	39,82±0,02
5	P5	AL+RC+KG	7,46±0,07	31,62±0,01
6	P6	AL+RC+CMC	7,25±0,02	22,40±2,72
7	P7	AL+KG+CMC	7,58±0,09	Not breaking

All values represent means ± standard deviation with, n = 3 and (p <0.05).

For sample P1 with sodium alginate, the breaking point of the film marked the value of 50 MPa registering the highest value compared to the other samples analyzed. However, in the case of samples P3 (AL + KG) and P7 (AL + KG + CMC) due to the superior elastic characteristics that these films possess, they did not break during the mechanical strength test. Sample P7 was the ideal mixture of sodium alginate hydrocolloids, Konjac gum and carboxymethylcellulose for a support matrix in which bioactive elements can be incorporated. The analyzed samples showed values of breaking strength between 22,5 MPa and 50 MPa, values similar to those obtained by Silva et al. (2009), in films based on pectin and alginate.

The individual rheological determinations of AL, CMC, RC and KG gels in Figure 3.5. indicated promising results of the elastic modulus for (AL) and (CMC) representing stable components whose linearity was constant over the entire strain range. In the case of samples (RC) and (KG), although the mechanical strength was superior to the previous samples, it remained stable only up to 1%, after which it started to lose significantly in value.

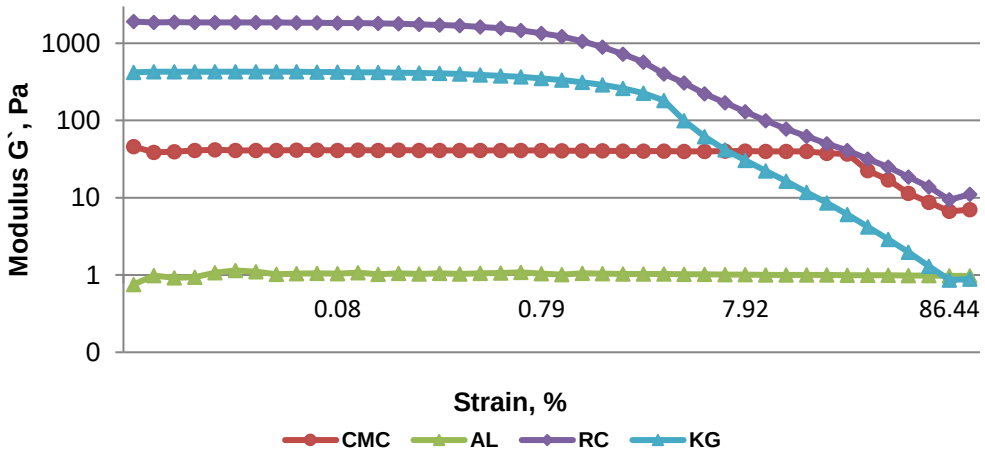


Figure 3.5. Variation of modulus of elasticity, G' for CMC, AL, RC and KG gels in relation to material strain. Strain sweep test.

In figure 3.6. the viscosity modulus, G" recorded linear values over the entire strain range for samples (AL) and (CMC). Precisely the linearity of the two samples suggests a possible synergy of them and their use in the manufacture of bioedible functional films. In the case of samples (RC) and (KG), modulus G 'indicated an increase in viscosity to 511 Pa at a strain of 3,97% for carrageenan and a viscosity value of 93 Pa at a strain of 6,3%, respectively for Konjac gum. Both materials were viscous, but their linearity on the strain range varied. However, such materials can be used in small dosages to obtain bioedible films.

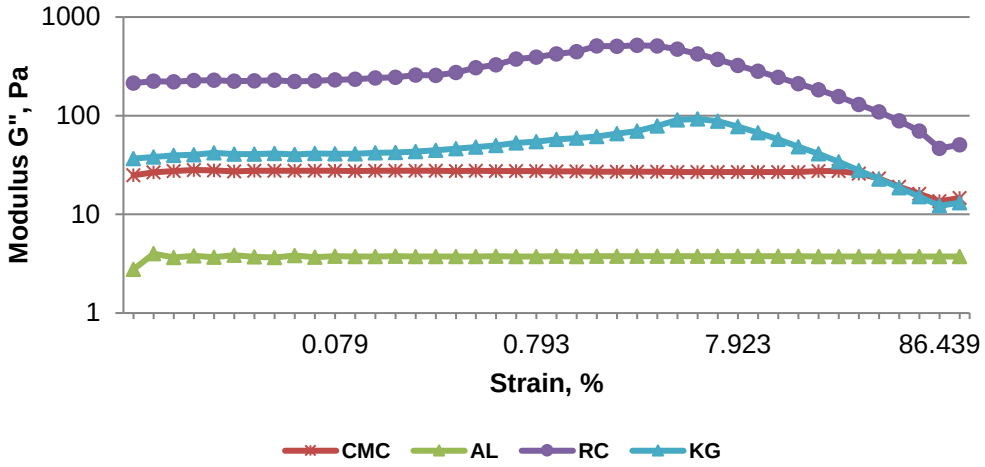


Figure 3.6. Variation of the viscosity modulus, G" for CMC, AL, RC and KG gels in relation to the strain of the material. Strain sweep test.

3.3.2. Antioxidant capacity of bioactive ingredients

The antioxidant capacity of the yeast extract and the fermented onion-based preparation was determined by a distinct, quantitative method based on DPPH activity. According to the results presented in Table 3.11., the antioxidant activity of the yeast extract had a significant value of 33,33% compared to that of the fermented onion-based preparation which had a value of only 21,85%.

Table 3.11. Antioxidant capacity for yeast extract and onion-based fermented preparation

Ingredient	Antioxidant capacity, DPPH-RSA (%)	DPPH, EC_{50} (mg/ml)
Yeast extract	33,33±0,25	0,88±0,21
Onion-based fermented preparation	21,85±0,15	0,60±0,13

All values represent means ± standard deviation with n = 3 and (p <0.05).

These two active ingredients can be introduced into the matrix of the film based on sodium alginate for their antioxidant and preservative effect and can provide a synergistic effect of protecting the product against the pro-oxidative action of external and internal factors. The EC_{50} value for the two extracts was 0,88 mg / ml and 0,60 mg / ml, respectively, which indicated acceptable values for these two ingredients. Similar results were recorded by Santas et al., 2008; Huang et al., 2009 and Singh et al., 2009.

3.3.3. Microbiological analysis of sodium alginate films

In figure 3.8. the interaction and influence of the active elements in the yeast extract and the onion-based fermented preparation on the viability of the *Bacillus subtilis* CU1 strain can be observed.

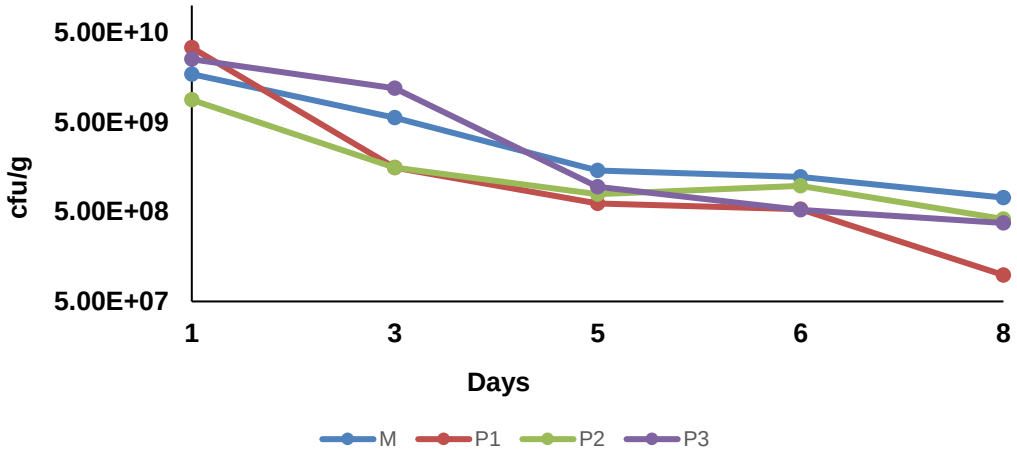


Figure 3.8. The influence of the active elements in the onion-based fermented preparation and the yeast extract on the viability of the probiotic strain *Bacillus subtilis* CU1 for 8 days at 4℃.

In the case of the control sample (M), the Petri dish was inoculated only with *Bacillus subtilis* CU1, and the viability of the cells for more than 7 days reached in this case a number of 7,2 × 10^8 cfu / g which may represent a considerably number, taking into account that there were no other interactions with related ingredients.

In the case of sample P1, which contained a mixture of *Bacillus subtilis* CU1 and onion-based fermented product, low values were obtained in the sense that a concentration of only 9,85 × 10^7 cfu / g was obtained. This can be explained by the use of the onion-based fermented preparation which can be characterized as one of the best antimicrobial agents of vegetable origin, with action similar to garlic. The fermented onion-based preparation can help inhibit and develop the strain of *Bacillus subtilis* CU1 and many other microorganisms due to the bacteriocins it contains, and which attack the bacterial cell membrane. These bacteriocins section the microbial membrane by creating pores which increase the permeability of tiny compounds, causing a rapid outflow of pre-accumulated ions, amino acids and in some cases adenosine 5-triphosphate (ATP) molecules. A major reaction occurs when the onion-based fermented preparation is used with the appearance of thyopropanal-S-oxide. The onion-based fermented product also contains antimicrobial phenolic compounds, proto-catechin acid and catechol (Montville et al., 2012; Ray et al., 2013).

For the P2 sample where a mixture of *Bacillus subtilis* CU1 and yeast extract was used, superior results were obtained in terms of cfu / g value compared to the P1 sample. Here the maximum value reached the number of 4,15 × 10^8 cfu / g, due to the yeast extract that potentiated the development of the *Bacillus subtilis* CU1 strain, which contains high amount of nitrogen required for the microbial metabolism. These results were possible due to a symbiotic relationship between the two bioactive ingredients; the yeast extract, due to the high content of B vitamins, is a growth factor for bacteria which, in turn, produce lactic acid creating an optimal pH for yeasts. Yeast extract contains at least 55% protein in the composition and is rich in glutathione. Glutathione can prevent

the degradation of important cellular components caused by oxygen-reactive species such as: free radicals, peroxide, lipid peroxide and heavy metals (Pompella et al., 2015).

In the case of sample P3, a mixture of *Bacillus subtilis* CU1, yeast extract and onion-based fermented product was used and an optimal result was recorded in terms of microbial population growth. This mixture had an intermediate point where the fermented onion-based preparation and the yeast extract act synergistically and potentiate the development of the *Bacillus subtilis* CU1 strain, reaching a value of 3,75 × 10^8 cfu / g.

Cell multiplication of the *Bacillus subtilis* CU1 strain in sodium alginate-based films with incorporated active elements was illustrated in Figure 3.9.

For formula (1), sodium alginate, konjac gum and carboxymethylcellulose were used as starting materials to obtain the bioedible film. In the case of sample 1A, the lowest values of all six determinations were obtained, reaching the number of 8,3 × 10^8 cfu / g after 8 days. This result can be attributed to the fact that sample 1A, the fermented product based on onion and yeast extract was inoculated with the smallest amount of *Bacillus subtilis* CU1.

In the case of samples 1B and 1C, the results obtained were with an improved logarithmic unit in terms of number of cfu / g, with values reached of 1,1 × 10^9 cfu / g and 1,3 × 10^9 cfu / g respectively. These similar results were much higher than those in sample 1A, due to the doubling of the amounts of active elements incorporated in the film matrix for sample 1B and the tripling of the amounts of active elements for sample 1C.

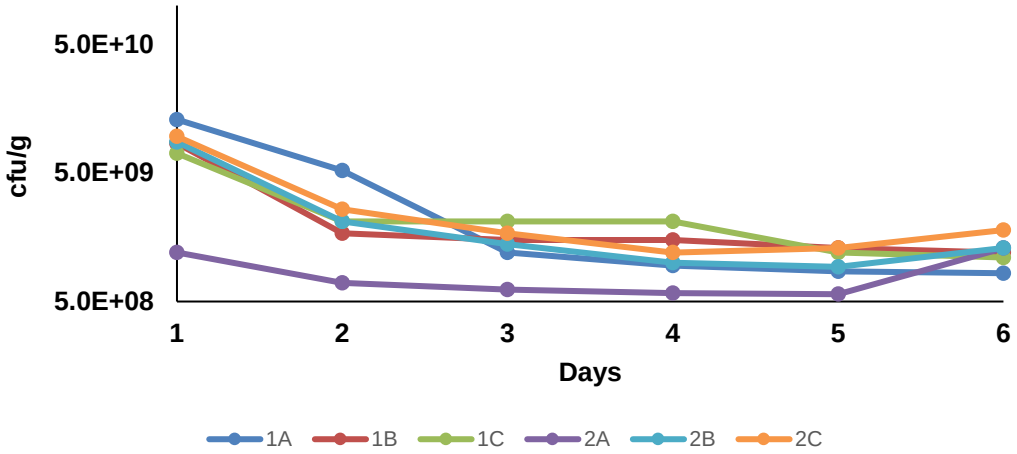
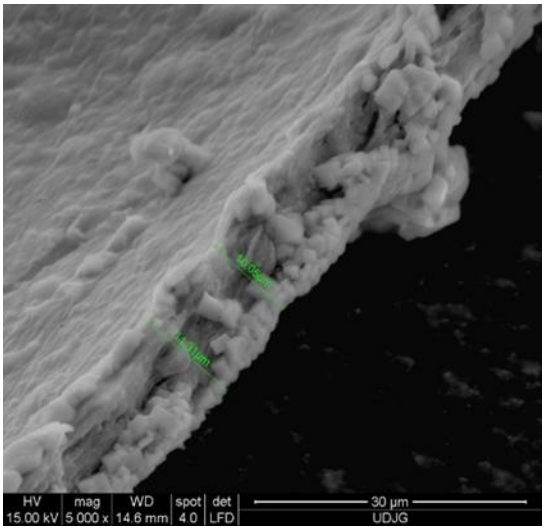


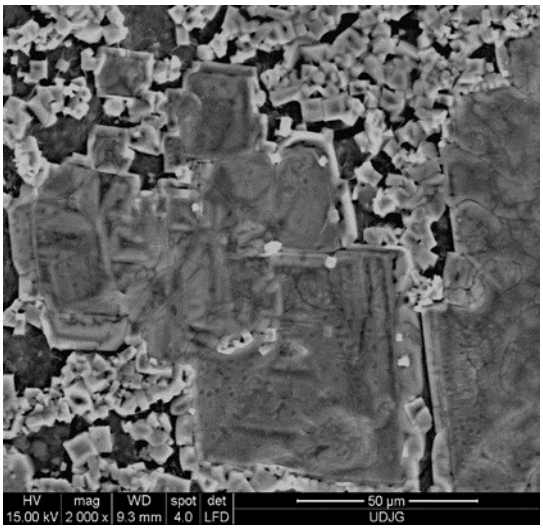
Figure 3.9. Multiplication of *Bacillus subtilis* CU1 in bioedible films based on sodium alginate with incorporated active elements.

The results for sample 1C, although the amount of bioactive ingredients was 50% higher than in sample 1B, were approximately similar. The logarithmic reduction was directly proportional to the increase of gallic acid concentration, which reveals the high antimicrobial activity of the onion-based fermented preparation. For formula (2), sodium alginate, carboxymethylcellulose, konjac gum and refined carrageenan were used to obtain the support matrix. In the case of samples 2A and 2B, close values were obtained, of 1,3 × 10^9 cfu / g and 1,4 × 10^9 cfu / g, respectively, after 8 days, which represent similar values to those obtained for the films stabilized in the formula (1). In the case of sample 2C, values of 1,8 × 10^9 cfu / g of *Bacillus subtilis* CU1 were obtained; normal values since this sample was inoculated with the largest amount of microorganism and bioactive ingredients. For both formulas, the top values were obtained in the case of samples 1C and 2C, respectively, for the maximum dosages of active elements.

Figure 3.10. represents a section of the bioedible film captured with SEM technology, where different layers of polymers can be observed incorporating the bioactive components.

Figure 3.10. Section images of bioedible films and representation of the structure of *Bacillus subtilis* CU1 colonies at SEM.





The surface of the film was irregular and rough in appearance, showing wrinkles, with a uniform, compact and dense structure. From the interactions of the chemical elements resulted salts that crystallized in a cubic form, with dimensions between 2,47-3,47 µm. Sometimes these crystals stick together and form uneven cross structures, as in Figure 3.10.

Images 1A, 1B and 1C in Figure 3.11., provide a clear perspective on the surface of Alginate-based films that incorporated the bioactive ingredients and how microorganisms developed by creating small snowflake colonies (1A) or overlapping between them in large cross-shaped colonies and irregular surface. These were evenly distributed in the film structure 1B and 1C.

Images 1A', 1B' and 1C' in Figure 3.11. were increased by 5000x and illustrated the population density of *Bacillus subtilis* CU1 which was homogeneously distributed. Sample 1A' was more sporadic in number, while samples 1B' and 1C' were very consistent; these were directly correlated with the amount of *Bacillus subtilis* CU1 inoculated in the recipe. For images 2A, 2B and 2C, the situation was similar to that described above in formula (1). Sample 2A', magnified to 10,000x, showed a heterogeneous exposure of the cells in the hydrocolloid film matrix. In this case, a thin layer of gel surrounded the walls of the stem shape of the grown crop.

Sample 2B' showed a large abundance of crystal structures with cube shapes and different sizes. For the 2C’ test, good details were obtained about the agglomeration and adherence of the pure culture. In this case, the reading was performed with a magnification of 2000x.

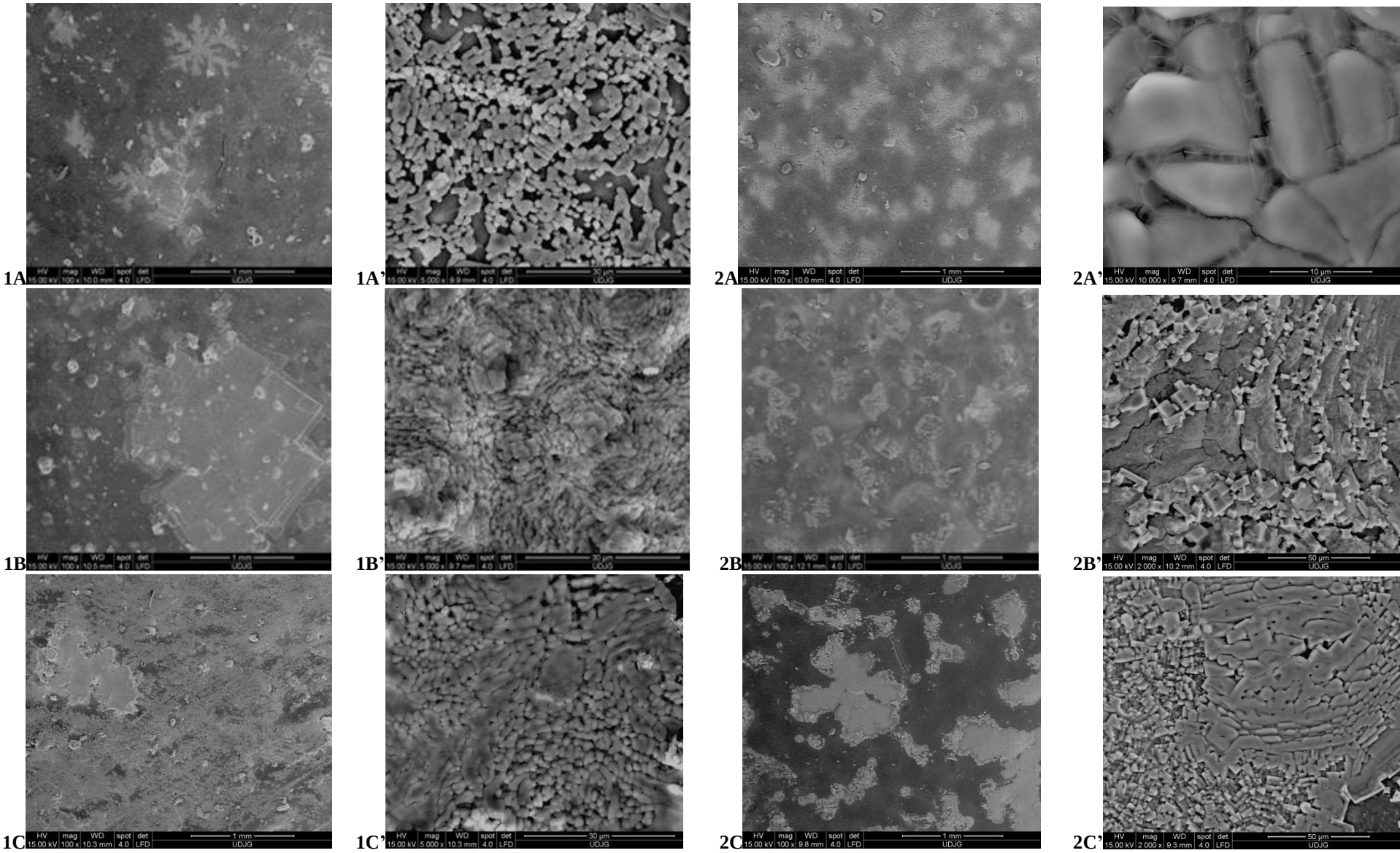


Figure 3.11. Representation of films incorporated with *B. subtilis* CU1 at different resolutions by SEM equipment

3.3.6. Permeability of bioedible films based on sodium alginate

In table 3.16. there was an increase in the thickness of the bioedible films based on alginate, directly proportional to the increase in the concentration of glycerol and oleic acid added to the film formula. The addition of glycerol as a plasticizer in the recipe can slightly influence the thickness of the film, while the addition of glycerol and oleic acid represents a much more limited increase in thickness. The thickness of the films in this case varies from 0,13 mm and has reached the value of 0,30 mm for films with a high glycerol content.

Table 3.16. The influence of glycerol and oleic acid concentration on the thickness of bioedible films based on sodium alginate.

Sample	Glycerol, (g)	Oleic acid, (g)	Film thickness, (mm)
M	0	0,00	0,17±0,045
P1	0,675	0,00	0,22±0,015
P2	0,9	0,00	0,29±0,005
P3	**1,125**	0,00	**0,30±0,035**
P4	**0,675**	**0,675**	**0,13±0,01**
P5	0,9	0,9	0,15±0,01
P6	1,125	1,125	0,15±0,015

The thickness values represent means ± Standard Deviation, n = 3.

Water vapor permeability

According to the international standard ASTM E96, the method of vapor transfer by using cups / glasses can be described in 2 ways:

a. **The method of cups filled with siccative. The transfer of water vapor is done from the external environment to the inside of the cup.**

The films obtained by the wet method had a permeability to medium water vapor, with values up to 38,348 g mm / kPa day m^2 at a film thickness of 0,30 mm, due to the formation of a three-dimensional polymer network stabilized by the chloride solution. calcium, thus capturing water molecules that act as plasticizers in a crystalline configuration, reducing the number of intermolecular bonds in the polymer chain and facilitating the transfer of water vapor through the film (Pintado et al., 2010).

Water vapor permeability ranged from 11,115 g mm / kPa day m^2 and a thickness of 0,13 mm to a maximum of 38,348 g mm / kPa day m^2 and a thickness of 0,30 mm as shown in table 3.17. The incorporation of glycerol as a plasticizer in the film formula influenced the water vapor permeability of bioedible alginate-based films. An increase in WVP could be observed with an increase in the concentration of glycerol added to the recipe. The most obvious value of WVP was marked by P3b where at a maximum glycerol concentration of 1,124 g a permeability value of 38,348 g mm / kPa day m^2 was reached. Unlike the control sample M, where WVP has the value of 16,188 g mm / kPa day m^2 at the refrigeration temperature of 8°C, all permeability values for glycerol samples had values higher than this value (from sample P1a to sample P3a, with permeability values of 19,918 g mm /

kPa day m^2 and respectively 26,637 g mm / kPa day m^2). Gradual but differentiated increases resulted between samples kept at 8℃ and those kept at 22℃.

Table 3.17. Permeability parameters of bioedible films based on sodium alginate by the method of drying cups at different temperatures.

Sample	t (℃)	Film thickness, (mm)	WVTR, (g/m^2day)	WVP, (g mm/KPa day m^2)
Ma	8	0,17	12,69	16,188
Mb	22	0,17	16,63	21,205
P1a	8	0,22	12,07	19,918
P1b	22	0,22	20,06	33,101
P2a	8	0,29	11,41	24,832
P2b	22	0,29	17,63	38,344
P3a	8	0,30	11,84	26,637
P3b	22	**0,30**	17,04	**38,348**
P4a	8	**0,13**	11,40	**11,115**
P4b	22	0,13	16,52	16,113
P5a	8	0,15	11,31	12,726
P5b	22	0,15	15,66	17,627
P6a	8	0,15	12,12	13,641
P6b	22	0,15	12,50	14,061

WVTR and WVP values represent means ± Standard Deviation, n = 3

In the case of maintaining the film in refrigeration conditions, the permeability values were much lower than in the case of maintaining the film at ambient temperature. For samples P4-P6, where the composition of the film based on alginate included both glycerol and oleic acid as plasticizers, we can note a modest and lower evolution of the permeability value with values reaching a maximum of 14.061 g mm / kPa day m^2 in the case of sample P6b. Compared to the control sample, this maximum value of the P6b sample was two units lower.

Thus, the glycerol-oleic acid mixture improved the permeability of alginate-based bioedible films. The reference values of the permeability of the samples with oleic acid and glycerol were for sample P4a of 11.115 g mm / kPa day m^2 at refrigeration temperature and for sample P6b of 14.061 g mm / kPa day m^2 at ambient temperature. The mixture of the two ingredients in this complex system contributed to the limitation of the intermolecular interactions in the structural matrix of the bioedible films based on alginate and, thus, the passage of moisture through the film was reduced / reduced.

b. Method of cups filled with distilled water. The vapors were transmitted from the inside to the outside, the inside is filled with distilled water.

In the case of the distilled water cup method, the permeability values varied negatively in the range of -90,100 g mm / kPa day m^2 and a thickness of 0,30 mm and -8,065 g mm / kPa day m^2 and a thickness of 0,15 mm (Table 3.18 .). The negative values of the parameters mean that the vapor transfer took place from the inside of the bucket to the outside. For the P1-P3 samples, a gradual decrease of the permeability value could be noticed with the increase of the glycerol content in the recipe, but this was inversely proportional to the increase of the film thickness.

Unlike control sample M, with a reference value of -10,284 g mm / kPa day m^2 at refrigeration temperature, only sample P3c had a relatively close value of -16,625 g mm / kPa day m^2, but here it also intervened. 0,3mm P3c sample thickness, almost double the control sample.

For control Md, with a reference value of -47,390 g mm / kPa day m^2 at ambient temperature, the only sample close in value was P1d, of -53,788 g mm / kPa day m^2, in this case the film thickness and the amount of glycerol in composition worsening the permeability parameters. For P4-P6 samples, the permeability values improved significantly due to the combination of glycerol with oleic acid in the film composition.

Table 3.18. Permeability parameters of bioedible films based on alginate by the method of cups filled with distilled water at different temperatures.

Sample	T, (℃)	Film thickness, (mm)	WVTR, (g/m^2day)	WVP, (g mm/kPa day m^2)
Mc	8	0,17	-8,06	-10,284
Md	22	0,17	-37,16	-47,390
P1c	8	0,22	-13,22	-21,811
P1d	22	0,22	-32,59	-53,788
P2c	8	0,29	-13,24	-28,794
P2d	22	0,29	-32,60	-70,929
P3c	8	0,30	-7,39	-16,625
P3d	22	**0,30**	-40,03	**-90,100**
P4c	8	0,13	-13,65	-13,314
P4d	22	0,13	-33,64	-32,807
P5c	8	0,15	-15,24	-17,152
P5d	22	0,15	-37,39	-42,074
P6c	8	**0,15**	-7,17	**-8,065**
P6d	22	0,15	-36,13	-40,662

WVTR and WVP values represent means ± Standard Deviation, n = 3

The analyzed permeability values in this case did not exceed the value of -42,074 g mm / kPa day m^2. Unlike the Mc control sample at refrigeration temperature, an improved permeability value was visible for the P6c sample of -8,065 g mm / kPa day m^2. In this case the ratio glycerol: oleic acid was 1:1 and the film thickness was 0,15 mm, representing an optimal value for the data in Table 3.16.

For the control Md at ambient temperature the higher value as permeability was -32,807 g mm / kPa day m^2, corresponding to sample P4d, where the ratio glycerol: oleic acid was 0,68: 0,68 and the film thickness was 0,13 mm.

3.3.7. EDS and SEM analysis

a. Quantitative EDS analysis

The EDS analysis was performed to obtain information about the chemical constituents of bioedible alginate-based films with various additions of bioactive ingredients. The values of the constituent chemical elements of the film were noted in table 3.19. where the main elements were compared between the tests performed.

Table 3.19. Chemical composition of bioedible alginate-based films, in mass percentage for samples P1-P4.

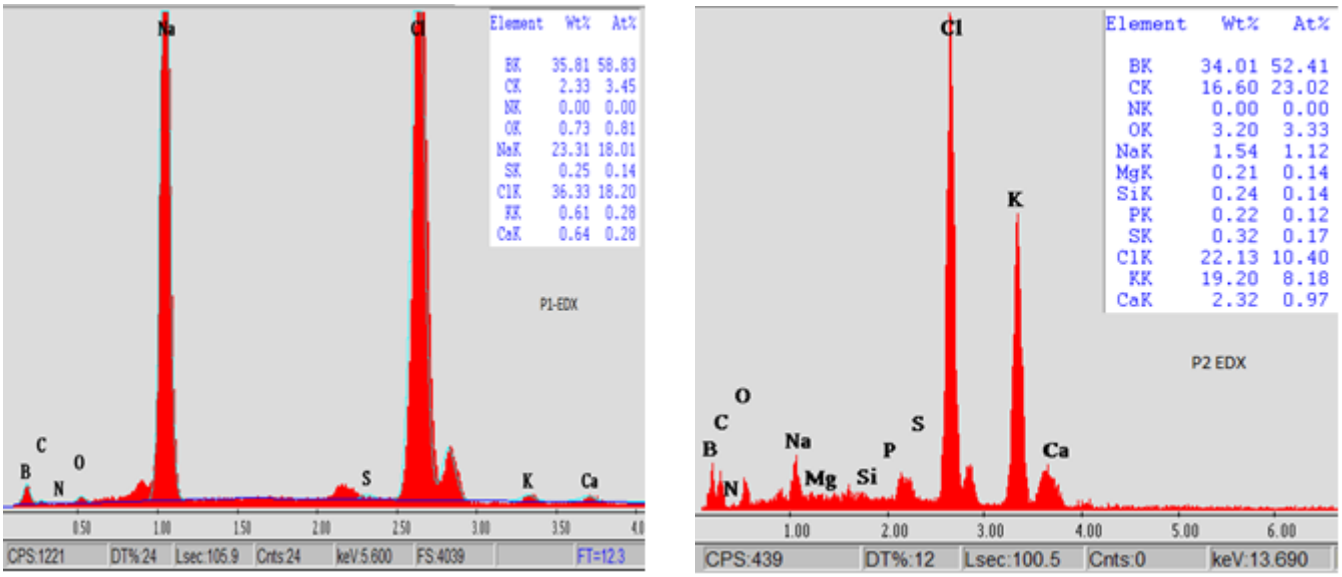
Chemical element	P1 (w/w)	P2 (w/w)	P3 (w/w)	P4 (w/w)
B	33,36±5,74	**33,676±5,56**	**24,308±4,56**	28,104±4,51
C	25,218±5,62	15,89±5,32	31,742±8,13	27,674±7,01
Cl	6,428±1,60	24,614±2,28	15,752±4,25	16,77±3,98
Na	5,912±0,78	14,132±4,97	4,256±5,23	2,42±3,61
O	17,766±2,27	5,228±2,38	12,924±3,14	11,472±2,78
K	5,622±2,64	0,392±0,19	0,612±0,36	4,624±3,93
Ca	4,994±1,10	4,862±3,32	7,72±3,05	6,758±1,30

The values of the analyzed chemical elements represent Mean ± Standard Deviation, n = 5
Probability p> 0.05, in rows for n = 5.

The superior chemical element observed in table 3.33. was the Boron for all samples taken in the analysis, with values ranging from 24,30 w / w to 33,67 w / w. Elements such as Na and Cl have also appeared in considerable proportions due to their presence in most of the added ingredients. Na ions were present in the composition of sodium alginate bound to the carboxyl group (–COONa) between various α-1,4 Glucuronate – Manuronate bonds. Na ions were also present in the composition of carboxymethylcellulose, but also in the fermented preparation based on onion and traces in the yeast extract. Ca and Cl ions came from $CaCl_2$ added for crosslinking alginate. Element C was found in most hydrocolloids used as their organic part. Low values for elements such as O and K were also noted in the composition of the analyzed films. Oxygen was present due to the incorporation in the various groups -OH or -COO- as a component part of the hydrocolloids used.

b. Qualitative EDS analysis

For the qualitative analysis using X - rays, it was necessary to identify the specific energy, in KeV, for the characteristic peaks of each chemical element (Figure 3.15.).

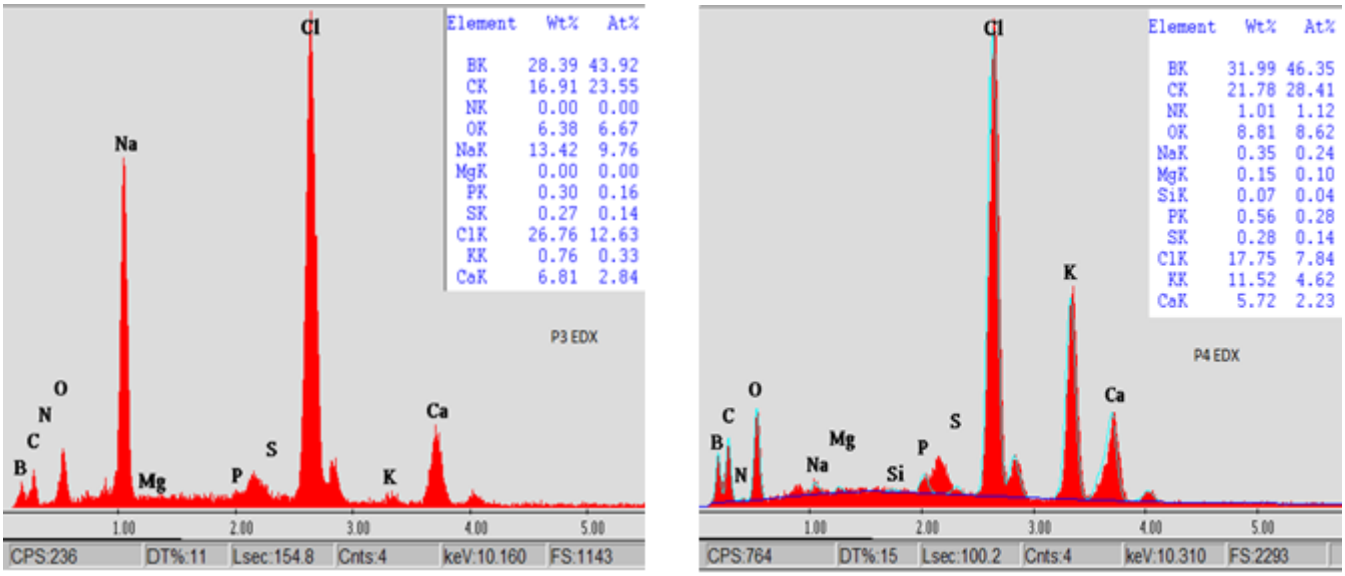


P1 P2

Figure 3.15. Schematic representation of the EDS Spectrum for samples P1 and P2.

Samples P1 and P2 had similar values of B, with an averages of 35% in the analyzed samples and, at the same time, similarities regarding the values of element Cl scored at 2,50 KeV, being obtained from $CaCl_2$ added for crosslinking the sodium alginate. For sample P1 the value of the element Na was specific for an energy of 1 KeV. It can be seen that, in the case of sample P2, due to the addition of a triple amount of yeast extract with a high mineral content of potassium, it can be noted an increased value of 19% in the energy peak around 3,4 KeV. The micrographic representations of samples P1 and P2 attest to the areas analyzed by the SEM and EDX equipment for which the above values were obtained.

For samples P3 and P4, the qualitative EDS analysis indicated common peaks for the values of the chemical element Cl around the energy value of 2,5 KeV (Figure 3.16.).



P3 P4

Figure 3.16. Schematic representation of the EDS Spectrum for samples P3 and P4.

In this case, similar values could be observed for both samples with tinted peaks for chemical element B at energy values <1KeV. Significant differences occurred in the case of sample P3 for the specific peak of element Na, which is abundant in this case, at the energy value of 1 KeV and in the case of sample P4 where there was a significant peak in element K determined at an energy value of 3,4 keV. This can be translated by the differences of the fermented preparation based on onion and yeast extract added in the two samples, in dosages of 0,5 g for sample P3 and 1,5 g for sample P4, respectively. These two bioactive extracts are rich in K and Ca elements notified by the peaks in the EDS schematic representation. Electron microscopy scans of samples P3 and P4 were performed to determine the structures of these alginate-based films. These were observed in the P3 SEM and P4 SEM samples above and correspond to the EDS readings next to them.

SEM analysis

Sample P1 (Figure 3.17.) illustrated a surface of the film crammed with an area of small salt crystals. This can be explained by a high concentration of Na and Cl elements with values of 23,31 w / w and 36,33 w / w respectively in the quantitative representation of EDS. The rest of the elements were mostly subunit with uniform values for the area in question. In the case of this test the image was magnified by 2000X. Due to the abundance of crystals on the analysis area, no cracks of the

film could be observed and it can be considered that the crosslinking of the alginate with calcium chloride was performed completely. The small dosage of the bioactive components in the recipe (yeast extract and onion-based fermented preparation) does not provide significant details for SEM analysis.

In the case of sample P2, the analyzed film area indicated the presence of larger crystals with high values for element C of 16,60 w / w, Cl with the value of 22,13 w / w and K with the value of 19,20 w / w. The high dose of the yeast extract and the fermented onion-based preparation in the recipe was much more evident here, with high values for K, specific to the yeast extract. The film showed cracks on the surface which may indicate either the analysis of a very thin area in terms of film thickness or an incomplete crosslinking of sodium alginate.

In the case of sample P3, it showed a crowded surface with a multitude of large crystals with values for Na and Cl elements of 13,42 w / w and 26,76 w / w, respectively. The sample showed no cracks on the surface.

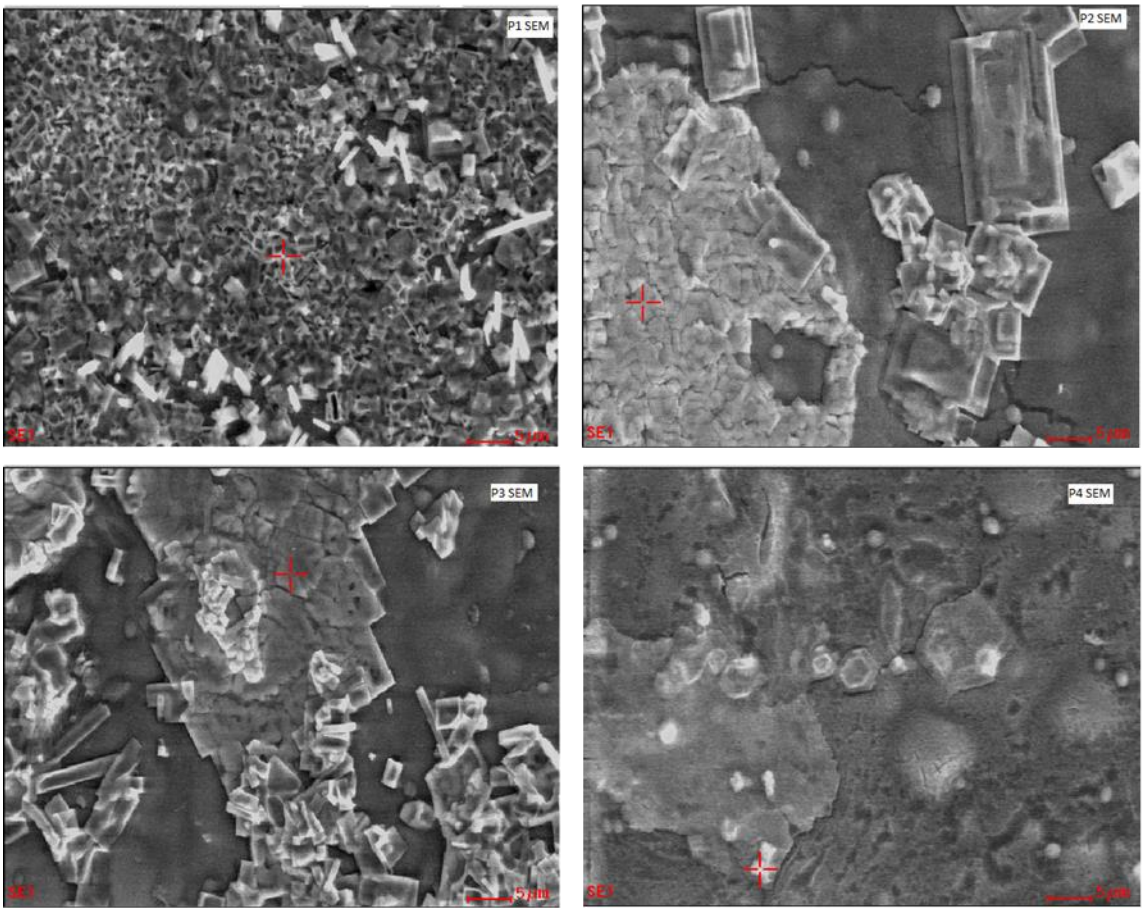


Figure 3.17. SEM representation of bioedible films based on alginate. Samples P1-P4.

The image was magnified by 2000X. The results showed that, like P1 sample, in this case, the low dosage of the active components made it impossible to detect the specific elements in the SEM analysis. In the case of the P4 sample, it showed a uniform distribution and no cracks, so an improvement in the mechanical properties of the film. In this case the value for element K was 11,32 w / w, an element specific to yeast extract.

3.3.8. FTIR analysis

In Figure 3.19, the energy peaks of the symmetrical elongated vibrations of the carboxyl group appear for the value of 1405 cm^{-1}. The second energy peak was highlighted around 1596 cm^{-1} and was associated with the vibrations of the asymmetric elongation of the carboxyl group (COO-) of sodium alginate. Jaya et al. (2009) and Huang et al. (1999) also found absorption bands for sodium alginate around 1320 cm^{-1} (CO group), 1120 cm^{-1} (CC group), 1090 cm^{-1} (CO group), 1020 cm^{-1} (COC group) and 950 cm^{-1} (CO group that has been attributed to the carbohydrate structure of sodium alginate). In our experiment, energy peaks of sodium alginate analyzed individually could be observed around: 1294 cm^{-1} (CO), 1080 cm^{-1} (CO), 1024 cm^{-1} (COC) and 947 cm^{-1} (CO), which are values similar to those mentioned above. FTIR spectra were used to determine the M / G ratio of sodium alginate for the characteristic absorbance peaks at 1024 and 1080 cm^{-1}. The mean absorption ratio (A1024 / A1080) for sodium alginate was 0,94. This result was similar to the results obtained by Vivian Florian-Algarin and Aldo Acevedo (2010).

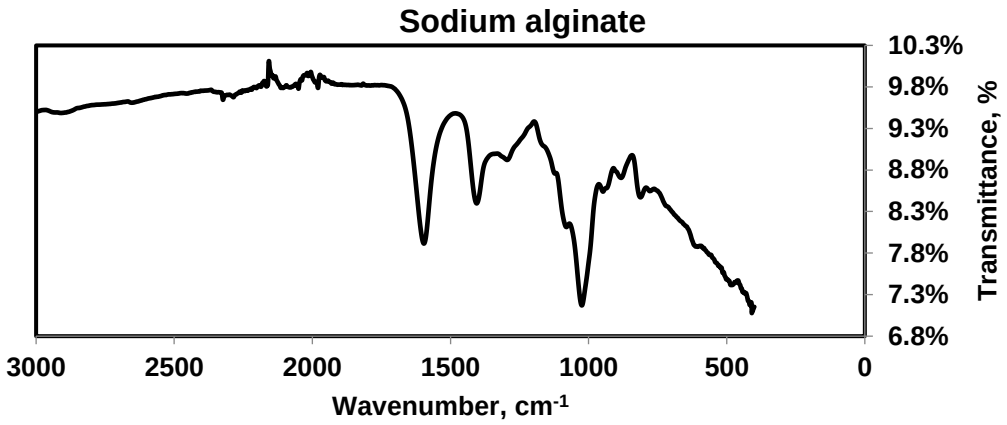


Figure 3.19. FTIR spectrum for sodium alginate used to obtain bioedible films analyzed as a single component.

According to Distantina et al. (2011), in the study of carrageenan using FTIR spectroscopy, the presence of a very strong band absorption was noted in the region 1210-1260 cm^{-1} (due to the S = O group of ester sulphates) and 1010-1080 cm^{-1} (attributed to the glycosides) present in all types of carrageenan. The other chemical groups are characteristic only for kappa-carrageenan, namely: 3,-6-anhydro-D-galactose at 928-933 cm^{-1} and D-galactose-4-sulfate at 840-850 cm^{-1}. The FTIR spectra for kappa-carrageenan also shows an absorption band in the range 840-850 cm^{-1}. In figure 3.20. specific strong bands can be identified for 1225 cm^{-1} (group S = O), 925 cm^{-1} and 1035 cm^{-1} (group C-O) and 842 cm^{-1} (group C-O-SO4).

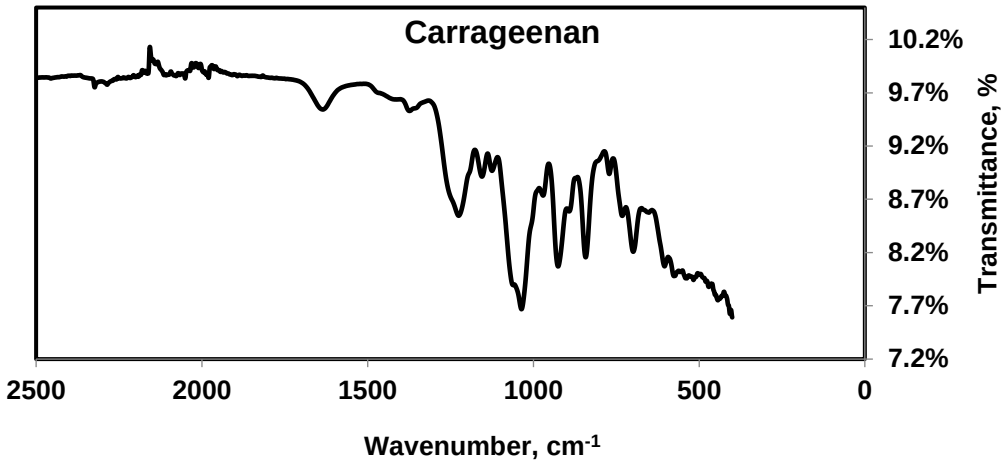


Figure 3.20. The FTIR spectrum for k-carrageenan used to obtain bioedible films analyzed as a single component.

Similar results were noted by Pereira et al. (2009), who mapped using FTIR-ATR spectroscopy about six distinct types of carrageenan to identify their composition.

In figure 3.21. representative bands were recorded, with intensities and / or integrated areas affected by carboxymethylation at values of 1321 cm^{-1} and 1588 cm^{-1}, attributed to the carbonyl expansion vibrations (group C-O). Similar results were obtained by Yuen et al. (2009) for absorption bands whose values reached peaks of 1315 cm^{-1} and 1605 cm^{-1}, respectively. In the case of peaks recorded at 1018 cm^{-1}, they can be assigned to groups (C-O-H).

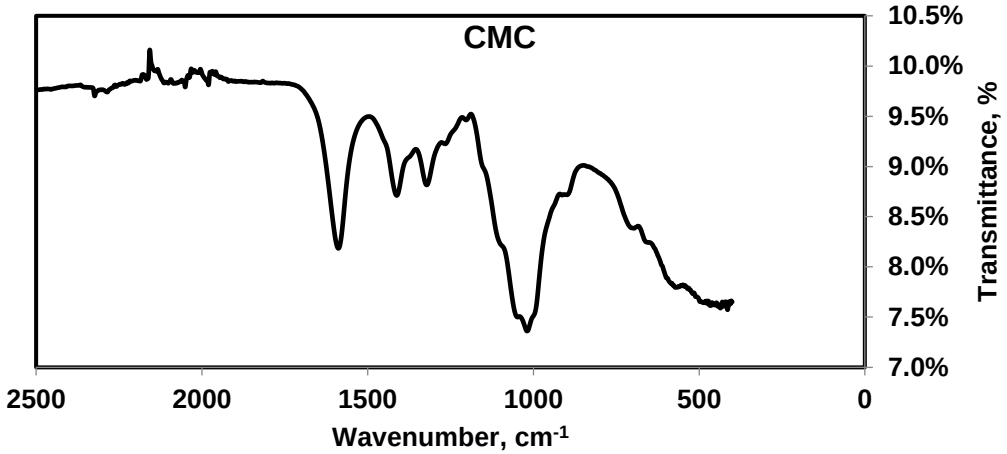


Figure 3.21. The FTIR spectrum for CMC used to obtain bioedible films analyzed as a single component.

In figure 3.22. the key characteristic peaks of Konjac Gum were represented with values at 3333 cm^{-1} (OH group), 2887 cm^{-1} (CH group), 1621 cm^{-1} (C = O group), 1148 cm^{-1} (O = O group) and 1013 cm^{-1} (CO group). Similar results were obtained by Huacai et al. (2006) and Yu et al. (2007), who also studied in detail the relationship between the component sugars for this polymer.

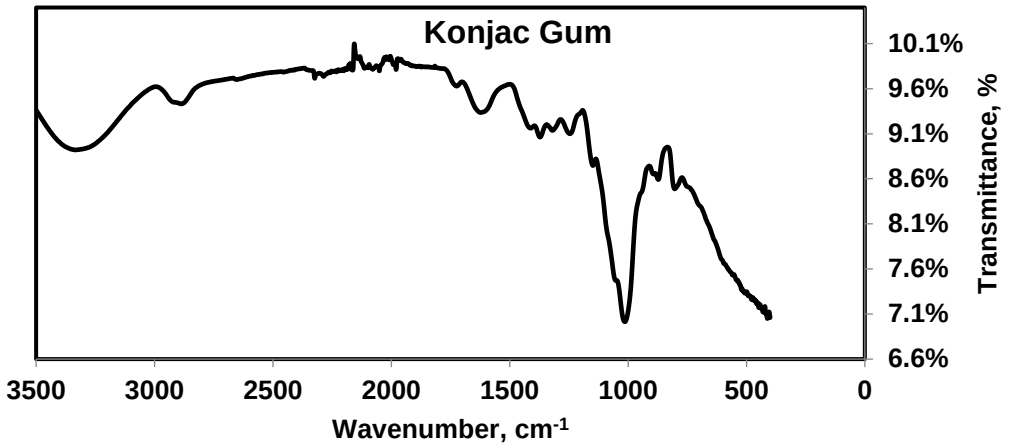


Figure 3.22. The FTIR spectrum for Konjac Gum used to obtain bioedible films analyzed as a single component.

In Figure 3.23 (M, 1A, 1B, 1C) and Figure 3.24. (M, 2A, 2B, 2C) The absorption bands for the FTIR spectra in the region 950-1200 cm^{-1} of the two recipes were illustrated. The presence of etheric bonds in polysaccharides can be verified by identifying intense absorption bands located in the fingerprint region, at wavelengths between 1000-1200 cm^{-1}. It was observed that the overlapping spectra showed identical absorptions in the fingerprint area, which proves with certainty that the analyzed samples contained compounds with structures of the same type.

Absorption bands around 1050-1080 cm^{-1} suggested the presence of C-O groups, groups specific to sodium alginate which is the main component used to obtain these bioedible films. Due to the low dosage of the bioactive ingredients incorporated in the film matrix, no considerable, clear differences could be recorded to indicate the presence of these compounds in the FTIR spectra of the analyzed films.

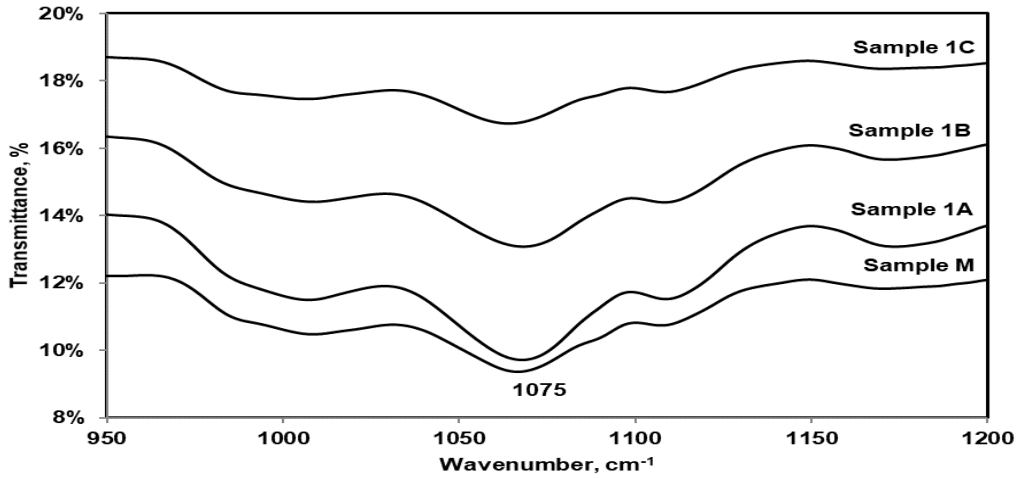


Figure 3.23. FT-IR spectra for alginate-based M, 1A, 1B and 1C film samples with incorporated bioactive ingredients.

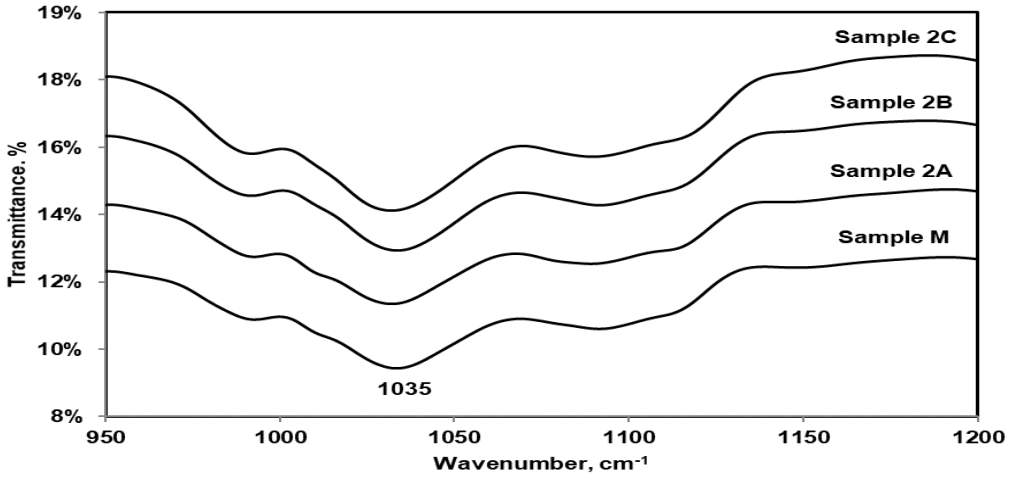


Figure 3.24. FT-IR spectra for alginate-based M, 2A, 2B and 2C film samples with built-in bioactive ingredients.

In the case of the two sets of illustrated recipes, minor differences can be observed regarding the addition of active compounds for each of the versions presented. This is due to the small amounts of organic acids, antimicrobials, metabolites and antioxidants present on the specific area of the film analyzed by infrared spectroscopy.

However, a significant variation could be observed between the films in Figure 3.23. and those in Figure 3.24. regarding the variation of the energy peaks located in the wavelength area specific to the C-O vibration groups with the value of 1035 cm^{-1} and the C-C groups specific to the value of 1075 cm^{-1}.

3.3.9. Experimental design

Regarding bioedible films based on alginate, the aim is to achieve a minimum value for WVP and WVTR in order to reduce the permeability of the membrane to vapors; we will choose the "Minimize" option for Optimizing this answer.

Figures 3.25. and 3.26. illustrate a comparison of the interactions between two components, before and after applying the recipe optimization to the selected water vapor permeability values.

Regarding the interaction between relative humidity and temperature, it was possible to observe from the experimental model a constant relative humidity value between 50-55%, but in the optimized model there is a significant change of the component thickness from the standard value of 0,22 mm to a recommended value of 0,15 mm. Such a recommended thickness is specific for the samples analyzed for versions P5 and P6, respectively, a high glycerol: oleic acid content between 0,9-1,12 g; WVTR values between 11,31-15,66 g mm / day m^2 and WVP values between 12,72 and 17,62 g mm / kPa day m^2.

Regarding the graphs with the interaction between temperature and film thickness in both cases an increase in water vapor permeability can be observed with increasing temperature. The analyzed temperature range is 8-22º C, and the relative humidity after optimizing the components reaches the value of 54,26%.

In this case, the value of water vapor permeability for optimal results reaches 12,76 g mm / kPa day m^2. In the case of the interaction between the relative humidity components and the film

thickness, variations of the relative humidity can be observed depending on the thickness of the analyzed film of 0,15 mm a temperature of 8º C and a relative humidity of 54,26%.

The analysis indicates a significant value from an experimental point of view by applying a predictability model that can be observed in table 3.20 and continued in 3.20.a. For R^2 values of 0.9680, the Quadratic model was the one suggested by the program and may be the best answer for the experiment in question. Another statistically interesting value was the value of P (probability), so that the chosen hypothesis is correct for values of p <0.05. In this case, the value of p <0.0033, indicated a relevant statistical experiment and whose hypothesis is confirmed.

Table 3.20. Representation of the recommended predictability model for optimization

Predictability table					
	Sequential	**Lack of Fit**	**Adjusted**	**Predicted**	
Source	**p-value**	**p-value**	**R-Squared**	**R-Squared**	
Linear	< 0.0001	0.0175	0.9296	0.9089	
2FI	0.0010	0.0884	0.9673	0.9558	
Quadratic	0.0033	0.3528	0.9846	0.9680	Suggested
Cubic	0.3528		0.9881		Aliased

Table 3.20.a. RH, temperature and thickness parameters for optimal film model

RH%: 50-55%	**Temperature: 8-22 °C**	**RH%: 50-55%**
Thickness: 0,225 mm----0,15 mm	RH%: 52,5%----54,26%	Temperature: 15 ^{0}C----8 °C

Figure 3.25. Components interaction from the experiment before Optimization.

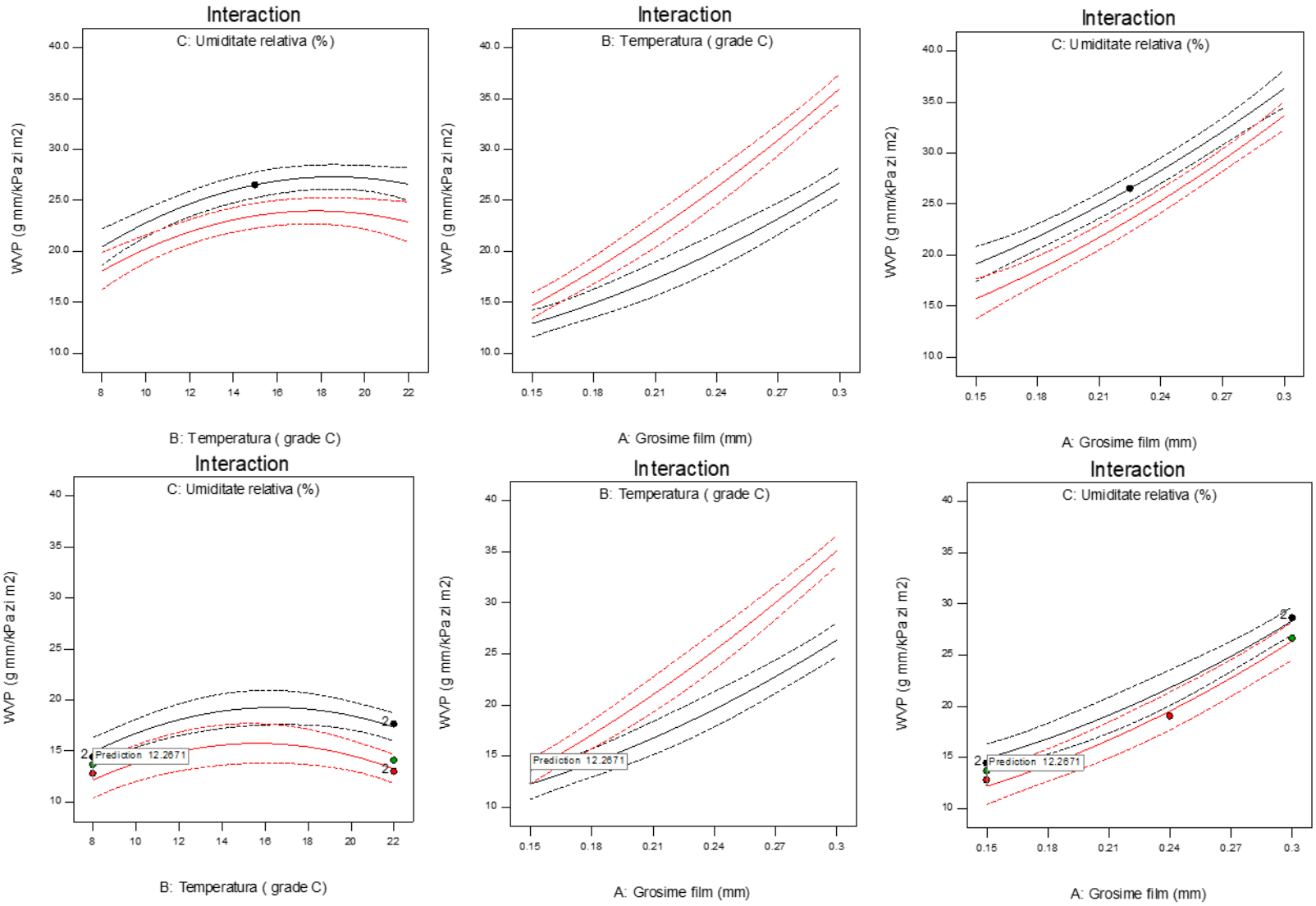


Figure 3.26. Component interaction after WVP Optimization for a minimum value of 12,76 g mm / kPa day m^2.

Experimentally, according to table 3.21., a Desirability factor around 0.972 can be reached, which indicates a value with a high probability to validate the analyzed hypothesis.

Table 3.21. Graph of the Optimal Desirability chosen by the program to improve the permeability of sodium alginate-based films.

Solutions								
No.	Thickness (mm)	t (°C)	RH (%)	WVP (g mm/kPa day m^2)	WVTR (g/m^2 day)	Desirability (<1)	w/o Interval	
1	0.150	8.000	54.279	12.265	11.071	0.972	1.000	**Selected**
2	0.150	8.000	54.303	12.260	11.060	0.972	1.000	
3	0.150	8.001	54.254	12.271	11.083	0.972	1.000	

Figure 3.27.a. illustrates the value, in which Desirability was the main factor analyzed, having as complementary factors the temperature and relative humidity recommended for maintaining the film. The relationship between causality and correlation can be identified by a direct connection between Desirability as a parameter analyzed and the other two factors, in the sense that, with increasing temperature, a decrease in Desirability can be observed (for example for t = 16℃ we have a value D = 0.75). The same is true for the relative humidity which, once reached the value of RH = 52%, will influence the value D = 0.78. Thus, the values of temperature and relative humidity had a direct effect on the desirability and at the same time on the permeability of the analyzed film. In the case of water vapor permeability, in Fig. 3.27.b. a predicted value of 12,267 g mm / kPa day m^2 can be observed as an optimization. The film thickness in this case was 0,15 mm, and the temperature and relative humidity parameters were directly dependent on the WVP values.

Thus, the permeability value increases in direct proportion to the temperature value (for example, if t = 16℃ is reached, then WVP = 18 g mm / kPa day m^2). In the case of relative air humidity, if RH = 51%, then WVP = 17 g mm / kPa day m^2. It can be concluded that the temperature, relative humidity and film thickness have a major influence on the permeability of sodium alginate-based films. In the case of WVTR (Fig. 3.27.c.), a predictable value is found around 11.06 g mm / kPa.

Similar to WVP, a direct correlation between temperature, film thickness and relative humidity can be identified for WVTR. Thus, with the increase of the values of these parameters, an immediate increase of the values for WVTR can be identified.

According to the predictions provided by the experimental model, the closest version that can be easily optimized to the ideal hypothesis can be given by the P5a test with the parameters presented in table 3.22.

Table 3.22. Bioedible alginate-based film test suggested as ideal by experimental modeling

Sample	Glycerol: Oleic acid (g)	t (℃)	Film thickness, (mm)	WVTR, (g/m^2day)	WVP, (g mm/kPa day m^2)
P5a	0,9:0,9	8	0,15	11,31	12,72

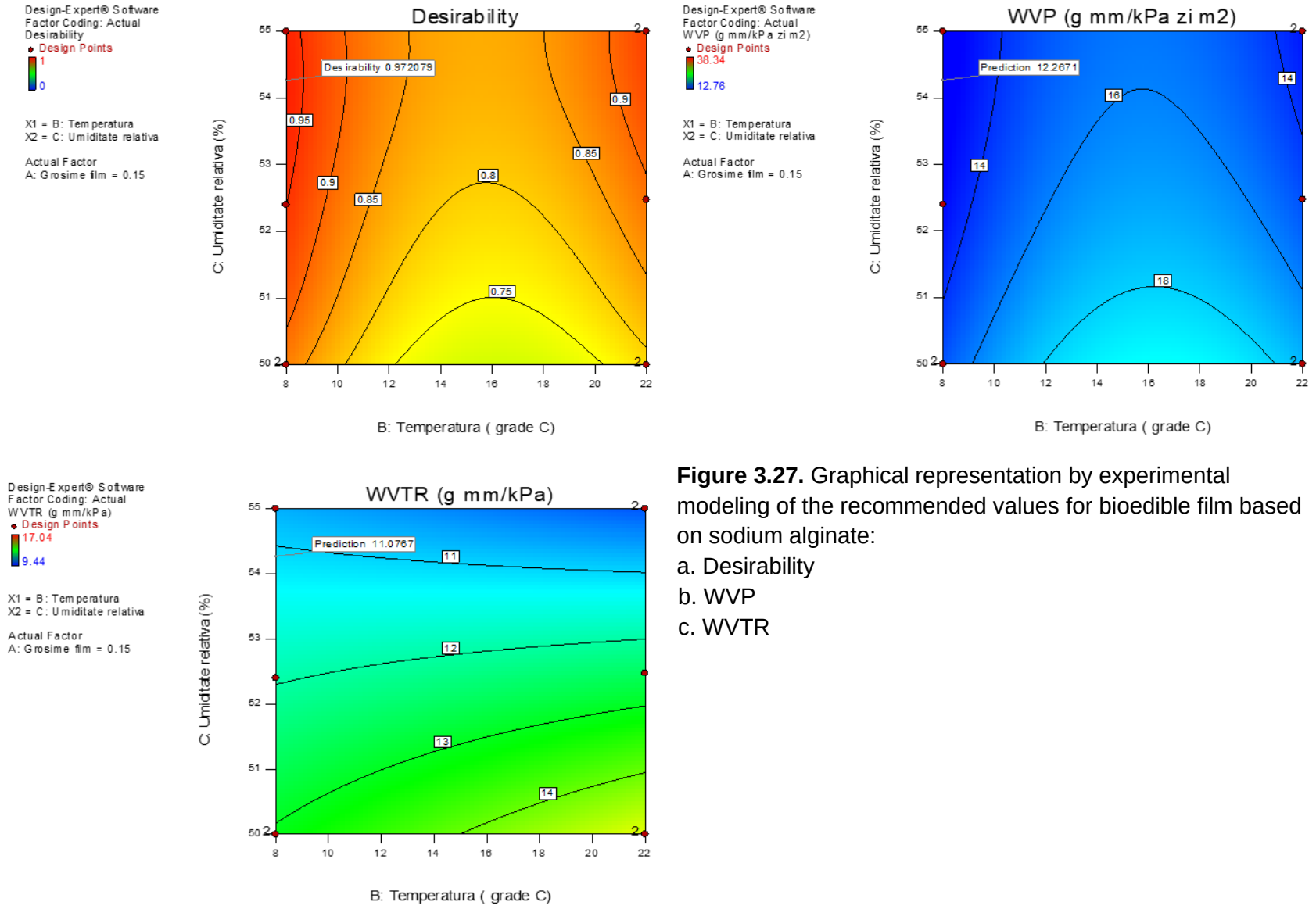


Figure 3.27. Graphical representation by experimental modeling of the recommended values for bioedible film based on sodium alginate:
a. Desirability
b. WVP
c. WVTR

3.4. Partial conclusions

- The optimal recipe, in terms of rheological behavior, with elastic properties and increased strength was established by the formula: 5% AL, 0,5% CMC and 0,5% KG and can serve as a support matrix for the various bioactive ingredients which will be incorporated in the final formula.
- The antioxidant capacity of the bio active elements such as: yeast extract and onion-based fermented preparation recorded values similar to those in other reference studies.
- The microbiological analysis of alginate-based films showed that, in both determinations made for individual bioactive ingredients and for alginate-based films incorporating these ingredients, the results had values higher than 10^8 cfu/g after the set time. In the case of individual bioactive ingredients, P2 sample (*Bacillus subtilis* CU1 + yeast extract) had a value of $4,15x10^8$ cfu/g which is a result to be considered during the 8 days of the study. In the case of alginate-based films with incorporated bioactive ingredients, formula 2 can be mentioned, AL + CMC + RC + KG and the mixture of (*Bacillus subtilis* CU1 + yeast extract + onion-based fermented preparation), which is the alternative with the best result of viable microorganism, reaching a number of $1,8x10^9$ cfu/g at the end of the study.
- For the Method of siccative cups, the experiment showed that the WVP had the optimum values between 11,11 g mm / kPa day m^2 and 12,72 g mm / kPa day m^2, at thicknesses between 0,13-0,15 mm and the temperature of 8℃, at which the ratio between the glycerol: oleic acid was 1:1. The films made from the mixture of hydrocolloids represented by sample P4a and P5a, were the optimal films for coating and protection against water vapor of a food product.
- For the Method of cups filled with distilled water, WVP had the best values located in the range -8,06 g mm / kPa day m^2 and -13,31 g mm / kPa day m^2, at thicknesses around 0,13 mm and 0,15 mm and the temperature of 8℃.
- Qualitative and quantitative EDS-SEM analysis can graphically illustrate the surfaces of the four analyzed samples while following the linearity and uniform or irregular structure of the films in question. For sample P1 the surface of the film was full of small crystals evenly distributed, without cracks at the surface of the film. Sample P2 showed an irregular surface with small and large crystals alike, but the film showed cracks / fissures at the lower level. Samples P3 and P4 showed irregular surfaces with crystals of different sizes without cracks in the film.
- The FTIR analysis suggested that the sodium alginate layers, representing the most abundant material in the recipe, establish synergistic interactions with the other added hydrocolloids, thus obtaining an optimal matrix for the active components.
- Experimental design. The optimization of the composition for the bioedible film mixture was performed with the help of an experimental model that helped in fine-tuning some key parameters regarding the analyzed components and their responses. According to the predictions provided by the experimental model the closest easily optimizable version to the ideal hypothesis is offered by the P5a test with the parameters> glycerol: oleic acid of 0,9: 0,9, optimum temperature of 8℃, film thickness of 0,15 mm, WVTR = 11,31 g / day m^2 and WVP = 12,72 g mm / kPa day m^2.

IV. Experimental production of a vegetable sausage filled in alginate membrane with incorporated bioactive ingredients

4.1. Introduction

This chapter includes the obtaining and application of the bioedible functional film on the surface of complex food matrices of vegetable sausage type. In this part of the thesis, microbiological analyzes were performed for two matrices: a vegetable sausage packed in collagen / polyamide membrane and the vegetable sausage packed in functional bioedible film. The physical, chemical analysis of both products was performed in parallel with the help of laboratory equipment.

In table 4.3. the production formula for a vegetable sausage with a balanced intake of protein, fiber, lipids and nutrients designed as a beneficial alternative to traditional animal products was presented.

Table 4.3. The formula for vegetable sausage in sodium alginate casing. Ingredients in mass percentage, %.

Ingredients	%
Water	58,00 %
Sunflower oil	13,00 %
Chickpea flour (23% protein)	10,00 %
Flax flour (40% protein)	9,80 %
Functional blend (pea protein and reffined carrageenan)	4,00 %
Preservative (onion-based fermented preparation, standardized)	3,00 %
Salt	1,50 %
Spice mix (pepper, sweet paprika, red beet powder)	0,70 %
Antioxidant (green tea extract, standardized)	0,03 %
Total	**100,00 %**

4.4. Materials and methods

4.4.1. Materials

The following materials with the corresponding coding were used in the experiments: chickpea protein flour (23% protein), manufacturer Naturis, Italy; flax protein meal (40% protein), Food Solutions Team manufacturer, Switzerland; sunflower oil, manufacturer Argus, Constanța, Romania; spice mix (pepper, sweet pepper, beetroot powder), producer Harke Group, Germany; custom functional blend (refined pea protein and carrageenan), manufacturers: AGT Foods, Canada and Danisco, Denmark respectively; preservative (onion-based fermented preparation), trade name: SLR100, manufacturer: Fi & S, Netherlands; antioxidant (green tea extract), manufacturer: Danisco, Denmark; [CMC] –sodium carboxymethylcellulose, manufacturer Ashland, USA; [AL] -Sodium alginate, manufacturer Caldic Ingredients, Rotterdam, The Netherlands; [RC] -Carrageenan Refined, BLG-Brilliant Gum Manufacturer, Shanghai, China; [KG] -Konjac mannan, manufacturer of Food Ingredients & Solutions, Netherlands; Selected culture medium Luria Bertani Agar, Miller, producer Titan Biotech Ltd., Rajasthan, India; [ED] - Yeast extract, producer Bio Springer Company, Maison Allfort, France; [PFC] - Onion-based fermented preparation, manufacturer Fi & S Company, Netherlands; Glycerol and Oleic Acid were used as plasticizers, manufacturer Sigma Aldrich, Germany; CaCl2 used as a crosslinking agent was supplied by Sigma Aldrich Company.

The investigations aimed:

- Determination of color, pH and spreadability for a vegetable rod in alginate membrane
- Comparative microbiological analysis for a vegetable stick in alginate and collagen membrane
- Sensory analysis for the two types of vegetable bars
-SWOT analysis

4.5. Results and discussions

4.5.2. Physical-chemical characterization

The effect of the addition of chickpea flour and flaxseed flour, as well as the collagen membrane coating of sodium alginate with incorporated bioactive ingredients, led to the production of a vegetable sausage with different physical, chemical properties than a similar pork product, beef or fish. In table 4.6. the chemical composition of the two vegetable protein flours is presented.

Table 4.6. Chemical composition of chickpea and flax flour used according to the data sheets in Annex 6

Ingredient	Dry matter, %	Ash, %	Lipids, %	Total proteins, %	Carbohydrates, %	Total fibers, %
Chickpea flour	91,4	3	7	22	58	11
Flax flour	92	5,1	8,8	40,3	37,7	33,8

Both varieties of flours had a high protein content as well as a total fiber content above average values. In the case of chickpea protein flour, the protein content reaches the value of 22%, in the case of flax protein flour, the protein content reaches an almost double value of 40,3%. The total fiber content for chickpea protein flour was 11% as opposed to the value of protein flour from flax, which reached 33,8%. For both protein flour assortments, relatively low values of lipid content can be observed in the range of 7-9%. However, flax seeds, flours and proteins have an important content of functional food ingredients due to their rich content of α-linolenic acid (ALA, omega-3 fatty acid), lignans and fiber.

Color determination

In table 4.7. the values of the identification parameters of the Frankfurter type vegetable bar product in alginate membrane and collagen membrane were presented. Incorporation of the bioactive ingredients into the alginate-based membrane had insignificant ($p > 0.05$) changes in brightness, red, and yellow.

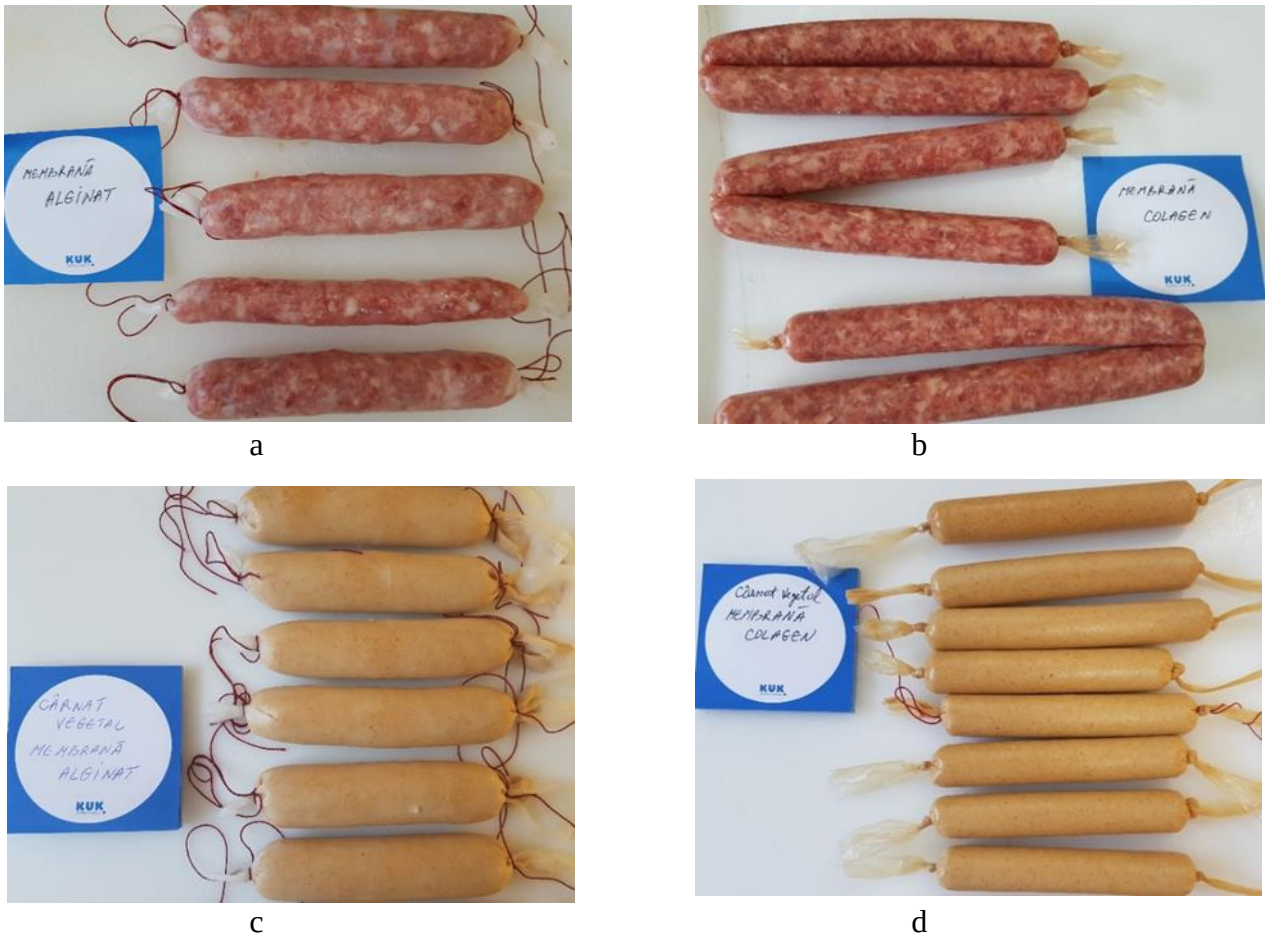
Table 4.7. Values of parameters such as color, spreadability, pH and dry matter for Frankfurter vegetable stick

Coating	Color			Spreadability, Pa	pH	d.m., %
	L*	a*	b*			
Alginate	64,7±0,010	6,05±0,025	27,72±0,035	63,09±0,070	6,20±0,140	45,19
Collagen	62,5±0,035	6,87±0,015	28,27±0,055	79,43±0,071	6,24±0,014	52,37

Values show means ± Standard deviation, n = 2, (p> 0.05).

In the case of the yellow color for the values of b*, the collagen membrane showed a slightly more pronounced shade with a value of 28,27, unlike the alginate membrane with a value of 27,72.

This slight color difference is also shown in Figure 4.2. (c and d) showing the detailed picture of the two assortments of membranes used on meat and vegetable composition.



a b c d

Figure 4.2. Frankfurter in membrane alginate and collagen pork (a and b); Frankfurter type vegetable sausage in sodium alginate and collagen membrane (c and d).

The yellowish, milky color of the sodium alginate-based membranes with incorporated bioactive ingredients did not influence the color of the finished product in the case of a vegetable

sausage, unlike the use of a normal collagen membrane. This is also due to the vegetal composition of the sausages which to some extent masks the quality and color of the membranes used.

Determination of spreadability

The results obtained after the analysis of the texture profile for the vegetable sausage obtained were presented in table 4. 7.. From the values recorded for the spreadability expressed as "flow threshold", we can see that it decreased significantly ($p < 0.05$), with replacement of collagen membranes with those based on sodium alginate. This is due to the low thickness and high-water vapor permeability of sodium alginate-based membranes. The same WVP behavior was observed for bioedible polysaccharide-based films in the study with increasing thickness due to the nonlinear nature of sorption isotherms. Water vapor permeability is a dynamic process. Water vapor spends more time in thick films causing a more careful approach to the equilibrium humidity value.

In figure 4.3. it is observed that the spreadability, expressed in Pascal, keeps a certain constancy up to a deformation of 40 Pa.

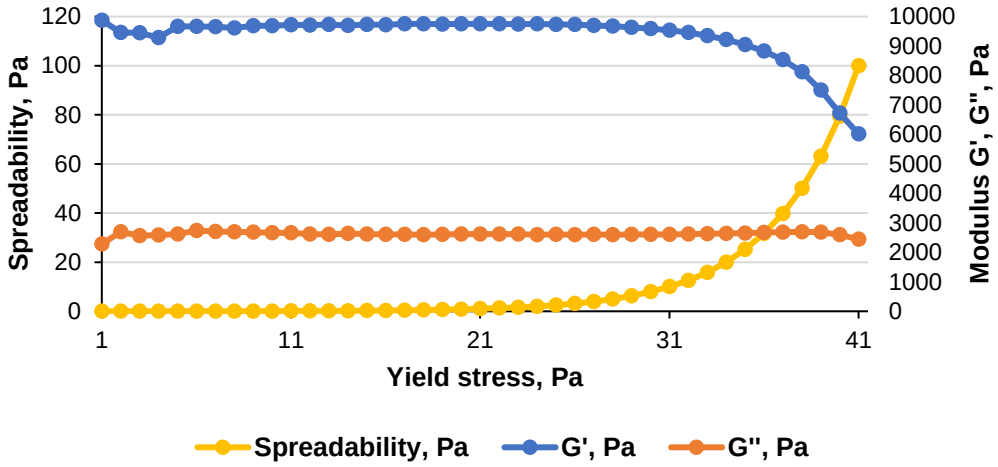


Figure 4.3. Spreadability expressed as "flow threshold" for the vegetable sausage in collagen membrane.

After the value of 40 Pa, a reduction of the elastic modulus G' was observed. The viscous modulus G" remained approximately constant as an evolution throughout the linearity domain.

Following the spreadability test, the critical oscillating force on the viscoelasticity domain was established. In figure 4.4. the spreadability reaches a maximum value of 63,09 Pa at the end of the linearity range.

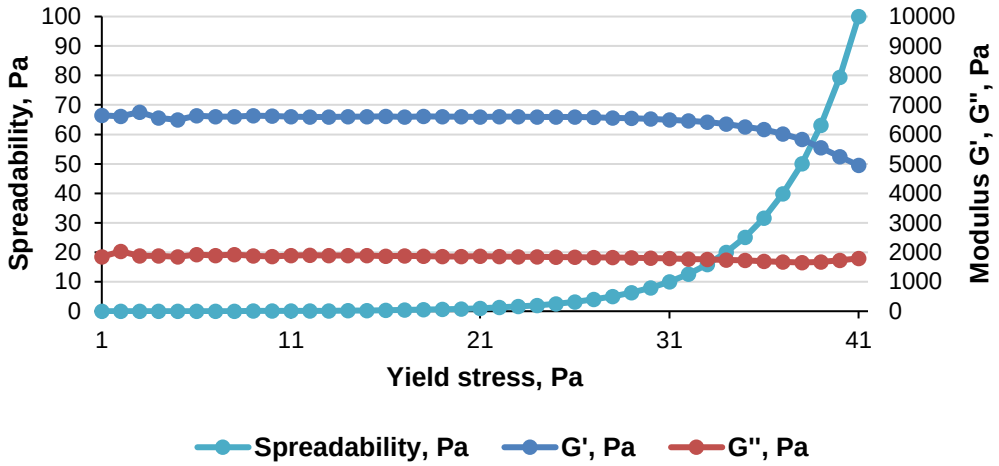


Figure 4.4. Spreadability expressed as "flow threshold" for vegetable sausage in sodium alginate membrane with incorporated bioactive ingredients.

Both the elastic modulus and the viscous modulus were constant throughout the test, recording lower values than the vegetable sausage in the collagen membrane.

Determination of pH

From the values recorded to determine the pH (Table 4.7), we can see that it decreased insignificantly ($p > 0.05$), with the change in the type of membrane for the spreadable plant product. Usually the membranes of sodium alginate or other polymers included in the product matrix have lower pH values, due to the substances used to obtain these assortments of membranes.

4.5.2. Comparative microbiological analysis for a vegetable sausage in alginate membrane and collagen membrane.

The results of the microbiological analyzes of the two assortments of vegetable sausage filled in alginate membranes and vegetable sausage filled in collagen membrane were presented in figure 4.5. (for yeasts and molds) and figure 4.6. (for mesophilic aerobic bacteria). In the first part of the analysis interval, up to day 5, the alginate membrane packed rod showed a higher value of cfu / ml compared to the collagen packaged rod. The CVB sample had a number of 4×10^2 cfu / ml as opposed to the CVC sample with a number of $1,8\times10^2$ cfu / ml. After day 5 of analysis the number of cfu / ml was higher for CVC than for CVB for the rest of the interval. Given the fact that the active membrane based on alginate had in its composition yeast extract with antioxidant role and the onion-based fermented preparation with preservative role, this can be explained in terms of active organic compounds present in the alginate membrane, compounds which inhibits the development of unwanted yeasts and molds. Although the initial dosage of these ingredients had a small value compared to the gel mass applied for the formation of the alginate membrane, it is more effective for this type of application.

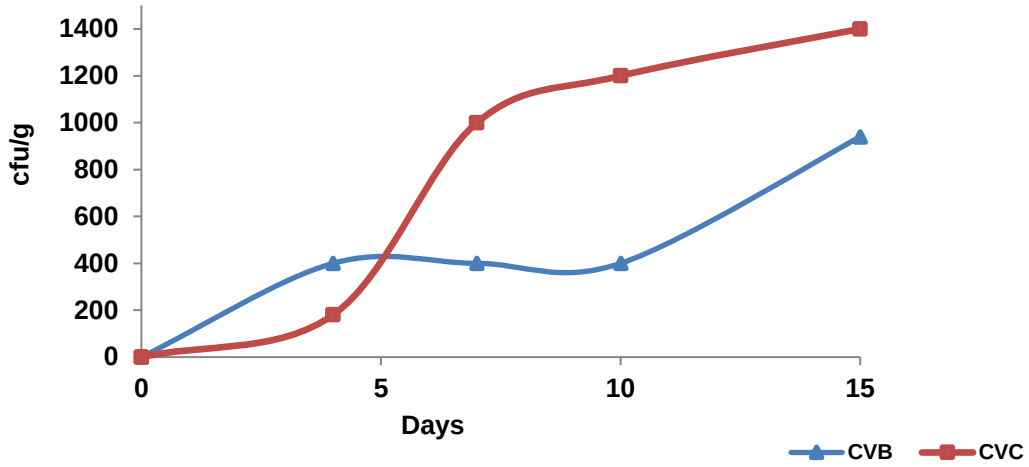


Figure 4.5. Number of yeasts and molds for the vegetable sausage in alginate membrane and collagen membrane in 15 days.

According to Mathenjwa et. al. (2012), the acceptable total microbial quality standard for fresh meat sausages is 10^6 cfu / ml, and for artificial membrane vegetable products of 10^3 cfu / g. There were some decimal differences on day 7 (4×10^2 cfu / ml for CVB and $1,0\times10^3$ cfu / ml for CVC), day 10 (4×10^2 cfu / ml for CVB and $1,2\times10^3$ cfu / ml for CVC) and on day 15 ($9,4\times10^2$ cfu / ml for CVB and $1,4\times10^3$ cfu / ml for CVC, respectively), differences which continued until on day 17 of the analysis.

The determination of mesophilic aerobic bacteria was illustrated in Figure 4.6. for both products packaged in alginate membrane and collagen membrane. There was a clear difference in the low value of cfu / ml bacteria for the product packaged in alginate membrane. Some decimal differences were recorded on day 4 ($5,0\times10^2$ cfu / ml for CVB and $1,5\times10^3$ cfu / ml for CVC) and on day 7 ($2,0\times10^3$ cfu / ml for CVB and $1,5\times10^4$ cfu / ml for CVC). After this reference, the situation continued in the same way until the 15th day with values ($1,7\times10^4$ cfu / ml for both CVB and CVC).

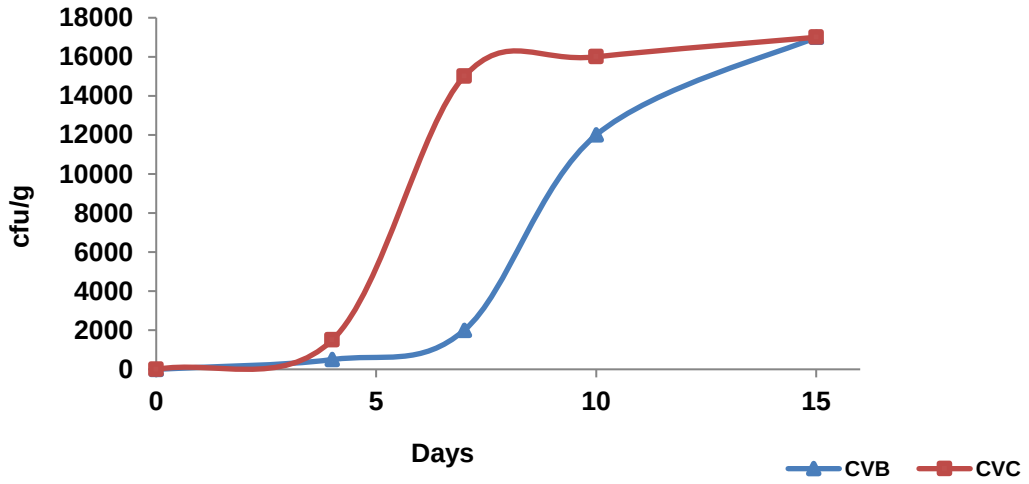


Figure 4.6. Number of mesophilic aerobic bacteria for vegetable sausage filled in alginate membrane and collagen membrane within 15 days.

Compared to the microbiological standards for such plant based products (SR EN ISO 2293: 1988 and SR EN ISO 21527-1: 2008) we can see that the original alternatives designed in this study

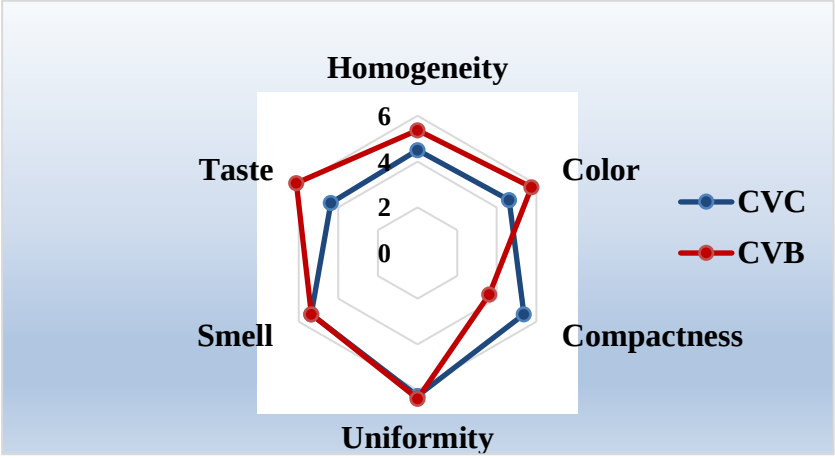
are microbiologically compliant, have a long shelf life and even after 15 days of refrigeration storage, the bacterial microbiota being below 5×10^4 cfu / ml. In addition, the absence of coliforms is noted throughout the analysis.

4.5.4. Sensory analysis - Scalar test

The results of the sensory analysis were presented in Figure 4.7.. The tasting session of the samples took place in the middle of the shelf life of both products which were kept refrigerated. CVB samples showed a more homogeneous and uniform paste structure opposed to CVC samples. On the surface, the membrane of both products was smooth, stretched and without wrinkles. Color and taste were evaluated with a lower score for CVC samples compared to CVB. However, in the section of the product the color was specific to the raw material from which the products were obtained, in the case of CVB slightly pinker due to the addition of paprika in the composition. The taste was specific to the raw material used (in this case chickpeas and flax) and suitable with a shade of salty and spicy.

Both CVB and CVC samples showed a constant uniformity of the paste along the entire length of the sausage. A score of 5,37 points was obtained by the CVC samples for the compactness attribute, these being much better coagulated, dense, without free particles compared to the CVB sample with a score of 3,62 points, which can also be associated with lower spreadability of this sample. The smell was specific to the heat-treated vegetable product for both samples due to the technological process of pasteurization to which they were subjected.

The tasting team's observations indicated significant differences in terms of aftertaste attributes and taste of foreign ingredients, respectively. Thus, the CVC sample showed an off taste of soy, bitter with shades of milk and egg felt during chewing the sample. At the opposite pole, the CVB sample was associated with a pronounced vegetable taste with shades of paprika and spices felt during chewing the sample.



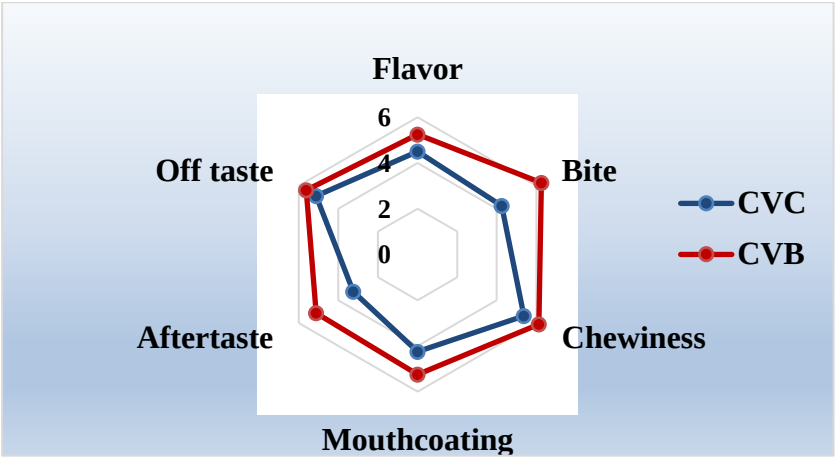


Figure 4.7. Key sensory attributes of samples encoded CVC (vegetable sausage with collagen membrane) and CVB (vegetable sausage with alginate membrane)

In figure 4.8. the general impression of the tasting team was represented by a generalized score of all the sensory attributes for the CVC and CVB samples. As a total score, the CVB test had an average score of 5,52 and a higher uniform value for 9 sensory attributes as opposed to the commercial test, CVC, which recorded an average score of 4,77 and a higher value for only two sensory attributes.

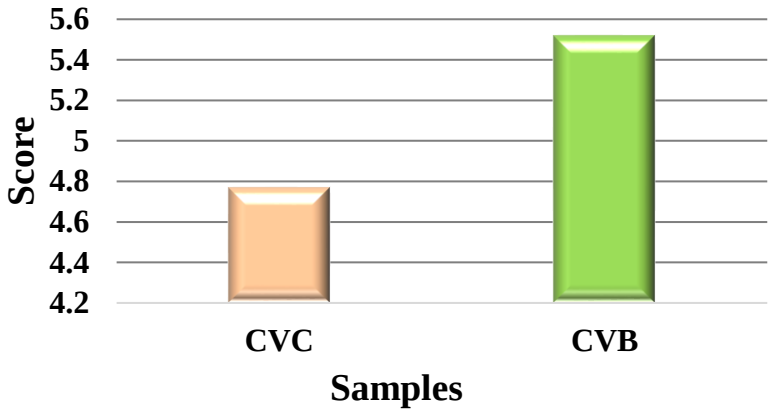


Figure 4.8. Overall impression of the tasting team for CVC and CVB samples.

4.5.5. SWOT analysis of vegetable bar in sodium alginate membrane

a. Strengths

• Efficiency and control of the vegetable raw material used both to obtain the composition of the product and to obtain the vegetable membrane,

• Obtaining a Frankfurter plant-based product with organoleptic and sensory characteristics like meat,

• Packing in plastic trays under modified atmosphere that allow a longer period of validity, transport and easy handling,

• Low costs and constant quality of alginate-based membranes as opposed to natural casings.

b. Weaknesses

• The equipment required for the formation of alginate-based membranes has high costs and is difficult to depreciate (Two filling machines synchronized with each other from Vemag or Handmann),

• The need to pay more attention to the settings of the filling / forming machines for the hydrocolloid gel necessary to obtain these membranes.

c. Opportunities

• New / innovative products aimed at a certain niche of consumers interested in exclusively vegetable products,

• Versatility in production, these membranes being used both for sausages and for raw dried salami or various vegetable products,

• Products currently not exploited to their true potential.

d. Threats

• Presence of additives on the product label (sodium alginate is labeled as number E according to current European legislation),

• Consumer reluctance to the type of plant membranes.

4.6. Partial conclusions

- Due to the composition of vegetable proteins used, the color changes were insignificant ($p > 0.05$) for sodium alginate membranes and collagen-based membranes.
- Regarding the spreadability of the products, expressed as "flow threshold", statistically significant differences were obtained ($p < 0.05$), due to the thickness and permeability of different water vapor recorded between the analyzed samples. Thus, the spreadability for the collagen membrane plant product reaches a maximum value of 79,43 Pa, in contrast to the spreadability of the alginate membrane plant product which reaches a spreadability of only 63,05 Pa (table 4.7.), Being softer, more inconsistent than the collagen membrane sample.
- The pH difference between the analyzed samples reveals a statistically insignificant value ($p > 0.05$) between the two products.
- In the case of microbiological analysis for the determination of yeasts and molds, in the case of both types of membranes some decimal differences were recorded on day 7 (4×10^2 cfu / ml for CVB samples and $1,0\times10^3$ cfu / ml for CVC samples), on day 10 ($4,0\times10^2$ cfu / ml for CVB and $1,2\times10^3$ cfu / ml for CVC) and on day 15 ($9,4\times10^2$ cfu / ml for CVB and $1,4\times10^3$ cfu / ml for CVC, respectively). In the case of microbiological analysis for the determination of mesophilic aerobic bacteria, some decimal differences were recorded on day 4 ($5,0\times10^2$ cfu / ml for CVB and $1,5\times10^3$ cfu / ml for CVC) and on day 7 ($2,0\times10^3$ cfu / ml for CVB and $1,5\times10^4$ cfu / ml for CVC). After this reference, the situation continued in the same trend until day 15 ($1,7\times10^4$ cfu / ml for both CVB and CVC). In addition, no undesirable impact was observed on the quality factors of plant products at the end of the 15 days of validity.
- Sensory, the general impression of the tasting team noted by a generalized score a total score for the CVB test of 5,52 points and a higher uniform value for 9 sensory attributes (homogeneity, color, uniformity, taste, flavor, chewing, roundness taste, off taste, aftertaste). For the CVC test, a total score of 4,77 points and a higher value was recorded for only two sensory attributes (compactness and smell).

- The SWOT analysis concluded several advantages and disadvantages specific to plant-based products coated in sodium alginate.

V. General conclusions, perspectives and personal contributions

General conclusions

The research carried out within the doctoral thesis aimed at obtaining an bioedible film with incorporated bioactive ingredients. Four hydrocolloids were selected in the experiment: sodium alginate, carboxymethyl cellulose, carrageenan and Konjac gum.

- The rheological characterization of each hydrocolloid studied, to establish the optimal composition of the film, highlighted the following aspects:
- From a rheological point of view, the optimal mixture of hydrocolloids to obtain a functional bioedible film is represented by the combination of sodium alginate, carboxymethyl cellulose and Konjac gum. Such a functional bioedible film ensures increased mechanical strength, a low value of water vapor permeability, homogeneous thickness and color and is also an ideal support matrix for the incorporation of bioactive elements such as yeast extract, onion-based fermented preparation and probiotic strain of *Bacillus subtilis* CU1.
- The formulation for obtaining bioedible films with incorporated bioactive ingredients, consists in several combinations of hydrocolloids selected in the study: AL + CMC, AL + CMC + KG, AL + CMC + RC + KG, to which bioactive ingredients were added: onion-based fermented preparation, yeast extract and a probiotic strain of *Bacillus subtilis* CU1.
- The viability and applicability of *B. subtilis* CU1 (CNCM I-2745), a probiotic intended for human consumption (Annex 2-Food grade certified. Probisis *BS*), was evaluated by performing a series of microbiological tests / analyzes involving the incorporation of this strain in the matrix of functional bioedible films.
- Microbiological analysis of alginate-based films showed that, in both cases of determinations made for individual active ingredients and in the case of determinations made for alginate-based films incorporating these bioactive ingredients, the viability of *Bacillus subtilis* CU1 culture had values higher than 10^8 cfu/g after the set time interval. In the case of individual bioactive ingredients sample P2 (*Bacillus subtilis* CU1 + yeast extract) had a viability of $4,15x10^8$ cfu/g, which is a promising result during the 8 days of the study.
- In the case of films based on alginate with incorporated bioactive ingredients formula 2 stands out, AL + CMC + RC + KG and the mixture of (*Bacillus subtilis* CU1 + yeast extract + onion-based fermented preparation), the solution with the better result in terms of microorganism viability, reaching a number of $1,8x10^9$ cfu/g at the end of the study.
- Experiments performed to determine the rheological characteristics, color, thickness, permeability of water vapor for sodium alginate films:
- The rheological characteristics of the films obtained using the AL + CMC and AL + CMC + KG mixtures were comparable, because of the use of similar mixtures of hydrocolloids in the film production formula. The composite film obtained showed remarkable elastic properties and increased strength. The optimal formula, in terms of rheological behavior, is based on the following mixture: 5% AL + 0,5% CMC + 0,5% KG and can serve as a support matrix for various bioactive ingredients which will be incorporated into the final formula.
- Regarding the color and thickness of the films based on sodium alginate, the addition of a large quantity of oleic acid, yeast extract and onion-based fermented preparation resulted in a change in color from light yellow to yellowish-white for samples 2A (oleic acid 0,925g,

glycerol 0,925g, OFP 1g and YE 1g) and 2B (oleic acid 1,025g, glycerol 1,025g, OFP 1,25g and YE 1,25g), at which the value of a* decreases from -1,58 to -1,60 and the thickness of the two films was around 0,13 mm. For films in which only glycerol, yeast extract and onion-based fermented preparation were added, the color intensified to dark yellow as the dosage of bioactive substances gradually increased.

- WVP and WVTR for bioedible films based on sodium alginate were evaluated according to the concentration of the solution, the thickness of the films and the type of plasticizer added to improve the properties of these films. In the case of the dry cup method, the experiment showed that WVP had the best values located between 11,11 g mm / kPa day m^2 and 12,72 g mm / kPa day m^2, at thickness between 0,13 -0,15 mm and a temperature of 8℃, at which the ratio between the glycerol: oleic acid was 1:1. The films made from the mixture of hydrocolloids represented by sample P4a and P5a, were the optimal films for coating and protection against water vapor of a food product. Regarding the distilled water cup method, WVP had the best values located in the range -8,06 g mm / kPa day m^2 and -13,31 g mm / kPa day m^2, at thickness around 0,13mm - 0,15 mm and the temperature of 8℃.
- Experimental analysis. The optimization of the composition of the bioedible film mixture was performed with the support of an experimental model, which helped in fine-tuning some key parameters regarding the analyzed components and their responses. The experimental design allowed the analysis of interactions between all components, respecting the desired proportions and properties of the material, so that the formulation of a recipe is improved before the preliminary tests, eliminating as much as possible the error. According to the predictions offered by the experimental model, it can be said that the closest version, easily optimized to the ideal hypothesis, is offered by the P5a test with the parameters: glycerol: oleic acid ratio of 0,9: 0,9; optimum temperature of 8℃, thickness 0,15 mm, WVTR = 11,31 g / day m^2 and WVP = 12,72 g mm / kPa day m^2.
  - In the case of microbiological analysis for the determination of yeasts and molds for both types of membranes, some differences of decimal order were registered; on day 7 values of 4×10^2 cfu / ml for CVB samples (vegetable sausage in alginate membrane) and $1,0\times10^3$ cfu / ml for CVC samples (vegetable sausage in collagen membrane), day 10 (4×10^2 cfu / ml for CVB and $1,2\times10^3$ cfu / ml for CVC) and on day 15 ($9,4\times10^2$ cfu / ml for CVB and $1,4\times10^3$ cfu / ml for CVC respectively). In the case of microbiological analysis for mesophilic aerobic bacteria, some decimal differences were recorded on day 4 ($5,0\times10^2$ cfu / ml for CVB and $1,5\times10^3$ cfu / ml for CVC) and on day 7 ($2,0\times10^3$ cfu / ml for CVB and $1,5\times10^4$ cfu / ml for CVC). After this reference, the situation remained constant as cfu / g on the 15th day, recording a value of $1,7\times10^4$ cfu / ml for both the CVB sample and the CVC sample). In addition, no undesirable impact was observed on the quality factors of alternative plant products at the end of the 15 days of validity.
  - From a sensory point of view, the score given for the overall impression (5,52 points) by the team of panelists for the CVB sample indicates a higher value for 9 sensory attributes (homogeneity, color, uniformity, taste, flavor, bite, chewiness, mouthcoating, aftertaste, and off taste). For the CVC test, a total score of 4,77 points and a higher value was recorded for only two sensory attributes (compactness and smell).

Prospects for further research

Following the accomplishment of the present study, the perspectives for further research are:

- Application of bioedible functional films with bioactive ingredients incorporated on a finished product such as vegetable sausage on an industrial production line and marketing the product at local, regional or country level.
- Studying the behavior of a functional bioedible film on other varieties of vegetable products such as fresh vegetable sausage, vegetable fantasy, etc.
- Promoting and highlighting the characteristics of these varieties of bioedible films in terms of the sustainability of the raw materials from which they are obtained.
- To study and diversify the type of hydrocolloids that can be integrated in such mixture to obtain superior bioedible films.

Personal contributions and scientific achievements

The originality of this study lies in the following:

- Detailed individual study of the main types of hydrocolloids (sodium alginate, carboxymethyl cellulose, Kappa-carrageenan and Konjac gum) currently used to obtain bioedible functional films in terms of their technological and rheological characteristics.
- Detailed individual study of the main bioactive compounds (onion-based fermented preparation, yeast extract and *Bacillus subtilis* CU1) that can be used for incorporation into the matrix of bioedible functional films based on sodium alginate.
- Studying the physical, chemical, technological and rheological behavior of bioedible functional films based on sodium alginate with incorporated bioactive ingredients.
- Obtaining by technological methods an alternative vegetable sausage in sodium alginate casing with incorporated bioactive compounds.
- Microbiological study for validation and verification of the shelf life in terms of development and multiplication of the number of microorganisms for the vegetable bar in sodium alginate membrane with bioactive ingredients incorporated over a period of 15 days, at a temperature of 8℃.
- Experimental design of sodium alginate-based films to obtain a flexible coating, medium in thickness, with a low water vapor permeability and with rheological properties similar to artificial membranes.

Dissemination of research results

The results of the research carried out during the doctoral studies were disseminated through the publication of representative articles for the approached field and communications at national and international scientific events.

A. Articles published in journals indexed in the Web of Science Core Collection

1. Ina Vasilean, Iuliana Aprodu, Marian Neculau, Livia Patraşcu. 2018. Effect of pulsed light treatment on germination efficiency of pulses. Scientific Papers-Series D-Animal Science, LXI (1), 266-274.

B. Articles published in BDI indexed journals

1. Marian Neculau, Vasilica Barbu, Giorgiana-Valentina Costea, Livia Pătrașcu, Camelia Vizireanu, Microbiological analysis and the antioxidant capacity of bioedible biofilms enclosing *Bacillus subtilis*, published in Scientific papers, Agronomy series, (2016), Vol. 59, no. 2, USAMV Iași

C. Abstracts published in ISI listed journals

1. Marian Neculau, Mihaela Alina Ceoromilă, Valentina Giorgiana Blaga (Costea), Gabriel Mustățea, Camelia Vizireanu, Structural and physicochemical properties of emulsified alginate-based film coatings for food products, International Conference "European Biotechnology Congress", Dubrovnik, Croatia, Journal of Biotechnology, 256S (2017) S44 – S116.
2. Giorgiana-Valentina Blaga (Costea), Marian Neculau, Livia Pătrașcu, Camelia Vizireanu, A comparison between low amplitude oscillatory shear and forced flow in determining honey rheological behavior, International Conference "European Biotechnology Congress", Dubrovnik, Croatia, Journal of Biotechnology , 256S (2017) S44 – S116.

D. Abstracts published in volumes of international conferences

1. Marian Neculau, Iuliana Aprodu, Daniela Borda, Livia Pătrașcu, Camelia Vizireanu, Physicochemical, rheological and experimental study of different biopolymers for obtaining functional bioedible films and coatings, 3-rd International Conference on Bio-based Polymers and Composites, BiPoCo, Szeged , Hungary, (2016), P37.

E. Communications at international scientific events

1. Ina Vasilean, Iuliana Aprodu, Marian Neculau, Livia Pătrașcu, Effects of pulse light treatment on germination efficiency of pulses. The international conference: Agriculture for life, life for agriculture, USAMV Bucharest, Romania, 2018.
2. Ina Vasilean, Iuliana Aprodu, Marian Neculau, Livia Patraşcu. The effect of physical pre-germination treatments on nutritional functionality of pulses. Bioavailability 2018 - Understanding the bioavailability of micronutrients and bioactive compounds for improved public health, September 10-13, 2018, Norwich, UK.
3. Ina Vasilean, Iuliana Aprodu, Marian Neculau, Livia Patraşcu. Germination as a tool for enhancing the nutritional value of pulses. The 8th International EuroFood Symposium - Mutatis mutandis in Food, 7-8 September 2017, Galaţi, Romania.

F. Communications at national scientific events

1. Marian Neculau, Camelia Vizireanu, Iuliana Aprodu, A biotechnological study of raw materials used for obtaining bioedible biofilms, Scientific conference of Doctoral Schools from University „Dunărea de Jos", Third edition, Galați, Romania, 2015.
2. Marian Neculau, Alina Ceoromilă, Livia Pătrașcu, Camelia Vizireanu, Biocompatibility of different hydrocolloids for obtaining bioedible films and coatings as packaging material. Doctoral schools conference, Galați, Romania, 2017
3. Marian Neculau, Iuliana Aprodu, Daniela Borda, Vasilica Barbu, Camelia Vizireanu, Bioedible films: technical, technological aspects and analytical methods for their characterization (Antioxidant and Microbiological aspects). Doctoral Schools from University „Dunarea de Jos", Third edition, Galați, Romania, 2018.

G. Adjacent activities

1. Ecotrophelia Europe:

a. Participation Ecotrophelia Europe 2017, Galați, Local Phase, May 20, 2017
First Prize --- HiProBar Product: Marian Neculau, Cristian Dragomir, Ramona Ifrim.
b. Participation Ecotrophelia Europe 2017, Suceava, National Phase, 3-4 July 2017
First Prize --- HiProBar Product: Marian Neculau, Cristian Dragomir, Ramona Ifrim.
c. Participation Ecotrophelia Europe 2017, London, International Phase, November 22-24, 2017: Marian Neculau, Cristian Dragomir, Ramona Ifrim.

2. Participation in adjacent research activities within the university:

a. Patent: Spreadable vegetable product and the process for obtaining it. Approved 30.06.2016. Inventors: Livia Pătrașcu, Iuliana Aprodu, Ina Vasilean, Marian Neculau. Accepted with no. PN-III P1-1.1-PRECBVT-2018-1205.

b. Research contract no. 173PED / 2017, code PNIII-P2-2.1-PED-2016-0155, entitled Development of new functional products based on germinated legumes, acronym ProPulse.

c. Participation in the National Exchange of Romanian Inventions: Conceived in Romania

d. Diploma of participation from the Ministry of Inventions for the patented product in Geneva: Spreadable vegetable product and the process for obtaining the same. Livia Pătrașcu, Iuliana Aprodu, Ina Vasilean, Marian Neculau. June 20, 2017.