MICROBIOLOGICAL ANALYSIS AND THE ANTIOXIDANT CAPACITY OF EDIBLE BIOFILMS ENCLOSING *BACILLUS SUBTILIS*

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Abstract

The aim of this research was to design edible biofilms on a basic matrix of sodium alginate (Al) and other hydrocolloids in which different proportions of onion extract (OE), yeast extract (YE) and *Bacillus subtilis (B. subtilis)* was introduced. These biofilms were evaluated from microbiological and antioxidant perspective.
The EC_{50} (mg sample/ml DPPH solution 6×10^{-5} mol/L) value was calculated, representing the active compound concentration which inactivates 50% of the total radicals DPPH. For the YE the EC_{50} value resulted 0.88 mg/ml and for the OE resulted with 30% smaller (0.60 mg/ml). The microbiological study targeted the influence of the OE and YE on cell viability of *B. subtilis* used as inoculum. A standardized inoculum of *B. subtilis* with a concentration of more than 10^{11} spores/g was used and a base culture of Luria Bertani Agar, Miller. After 48 hours of thermostating at 37° C the number of CFU/g resulted over 10^{10} in all experimental variations. A quantitative evaluation was made through indirect culture methods for a period of 8 days time in which the samples were preserved in refrigerated conditions at 4^{0} C. After analyzing the data we observed a constant behavior of the probiotic bacteria *B. subtilis* through all the conservation period. The *B. subtilis* + OE sample presented a reduction of CFU/g with one unit after 24 hours until the value of 9.85×10^{7} CFU/g was reached most probably due to the OE which has an antimicrobial effect. The best behavior over the preservation period was observed in case of *B. subtilis* + YE (4.51×10^{8} CFU/g) sample as we consider due to the YE which offers a good source of nitrogen and vitamin content that is necessary to maintain the viability of *B. subtilis* cells. Similar values have been obtained for *B. subtilis* + OE + YE (3.57×10^{8} CFU/g) with both extracts included onion and yeast that manifest a prebiotic effect for the bacterial cells.
This study showed that obtaining Al based films and incorporating distinct active ingredients with unique properties provide a „clean" solution and a future reference to healthy packaging materials. An edible biofilm which contains a mixture of YE, OE and a number of more than 10^{8} CFU/g probiotic bacteria even after 8 days in refrigerated conditions can be considered as an efficient solution of bio conservation and more than that could manifest a nutraceutical effect for consumer's immune system stimulation.

Key words: edible biofilm, *Bacillus subtilis*, yeast extract, onion extract, antioxidant activity.

The usage of bioactive extracts and specific probiotic microorganisms with the purpose of enhancing the intrinsic value of food is a rapidly spreading practice for food products. The research in food industry is focusing on incorporating active biological compounds in edible films with the goal of shelf life extension (Guilbert S. *et al*, 1996). These films are defined as thin and flexible layers obtained from animal or vegetal polymers which represent a functional matrix for active ingredients. Most biopharmaceutical production platforms are base on cell culture protocols, which can support high productivity while maintaining low operational complexity (Fike R., 2009). To be competitive, the food industry is challenging new techniques in developing or refining new processes to meet increasing market demands and reduce manufacturing costs while providing innovative healthy solutions. In this regard we imagined an edible biofilm matrix composed of four main hydrocolloids: Sodium Alginate (Al), Refined Carrageenan (RC), Carboxymethil cellulose (CMC) and Konjak Gum (KG), which should have superior individual properties and could influence the properties of the film in a positive way.

Furthermore, these films have proven to be excellent biomaterials for use as carriers of other natural active ingredients as antioxidants, antimicrobials, colorants, flavors, fortifying nutrients and spices: these additives improve the functionality of the packaging by bringing novel features (Pranoto Y. *et al*, 2005). In particular, addition of antimicrobial agents may enable

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extension of the shelf life and safety of packaged foods, by reducing (or even preventing) growth of pathogenic and spoilage microorganisms (Franssen R.L., Krochta J.M., 2003).

Moreover, their relatively low, but stable rates of diffusion from the packaging material onto the product assists in keeping the concentration of active ingredient relatively high as time elapses (Kristo E. *et al*, 2008). The antimicrobials more often incorporated in food packaging films are organic acids (lactic, acetic, citric and propionic acids), enzymes (lysozyme), bacteriocins (nisin), polysaccharides (chitosan) and some plant extracts and their essential oils (Cagri A. *et al*, 2004). Although extensive information on the antimicrobial properties of the above mentioned compounds are available in the literature, scarce data exists pertaining to the activity of OE and YE (Manab A. *et al*, 2011) when incorporated into Al films; and essentially no data at all regarding incorporation of *B. subtilis* in Al based films. Furthermore, a lack of information is apparent on the effect of those antimicrobial compounds upon the physical properties of Al based films. On the other hand, selection of an antimicrobial agent entails not only assessment of its effectiveness against target microorganism but also of interactions with the film-forming biopolymers; such interactions may indeed hamper the actual antimicrobial activity further to the characteristics of the film itself- both of which are key factors for development of commercially successful active films (Campos C.A. *et al*, 2011).

Therefore, the main purpose of this research was to obtain an optimum biopolymer film matrix which could be presented as a functional film that could protect a fresh meat product (fresh sausage) and provide an improved shelf life and a potential health benefit to the end product on an established period of 8 days in refrigerated conditions.

The effect of incorporating such compounds (natural preservative and a natural antioxidant source: onion extract and yeast extract) on the biofilm and the viable cell count of *B. subtilis* after 8 days were also assessed in this study.

MATERIAL AND METHOD

Materials

Probisis® *B. subtilis*, was obtained from LeSaffre Human Care, France and registered with CNCM no. I-2745; the selective medium of Luria Bertani Agar, Miller was provided by Titan Biotech Ltd., Rajasthan, India. The Yeast extract was acquired from Bio Springer, Maison Allfort, France and the Onion extract was supplied from Fi&S, Nethererland. High purity polymers from different EU suppliers were used in the experiment**:** (Carboxymethyl cellulose from Ashland, UK, type Blanose 7H9); (Sodium Alginate from Caldic Ingredients, Nd, type Ferwo F400); (Reffined Carrageenan and Konjac Gum from Fi&S, Netherland, type BLK 1120 and MRA). Glycerol used as plasticizer and $CaCl_2$ used as cross-linking agent were manufactured by Sigma-Aldrich. All materials were used based on certificate of analysis (CoA) and technical data sheets (TDS), reagent-grade or better and were used without further purification.

Characterization

The antioxidant potential of Yeast extract and Onion extract was assessed by determining the DPPH scavenging activity. A Spectrophotometer UV- Vis Zuzi was used to read the absorbance level at 517 nm of the 2 extract solutions. The surface morphology of Al based films and of composite films with active ingredients (Yeast extract, Onion extract and *B. subtilis*) was characterized by field-emission scanning electron microscope (SEM; Quanta 200) with 1.2 nm resolution and low voltage. Readings were made with magnification between 100x and 5000x.

Antioxidant activity

The DPPH radical-scavenging capacity of onion extract and yeast extract was determined according to the method described by (Gyamafi *et al*, 1999). For our study we used one milliliter of liquid onion extract (1 g powder for yeast extract) and 5 ml of 0.1 mM DPPH ethanolic solution and mixed them intensively. After this procedure we kept the solution in a dark space for 35 minutes at room temperature. The absorbance was then read at 517 nm by a spectrophotometer UV-Vis. The blank sample was prepared by replacing the extract with ethanol or water. The percentage of free radical scavenging activity was calculated with the formula described below:

$$\text{Scavenging activity, (\%)} = [1 - \frac{A_{517\ nm\ sample}}{A_{517\ nm\ blank}}] \times 100$$

The decrease in absorbance, which was induced by the tested sample and illustrated by a change in color from violet to yellow, was compared to that of the positive control used as standard. The EC_{50} value, representing the concentration of extract that requires the inhibition of 50% DPPH radicals, was also determined by calculation. The assay was carried out in duplicate and results were averaged.

Film and culture preparation system

The viability of *B. subtilis* was carried out on Luria Bertani Agar (LBA) medium using two complementary approaches: a first study was made by simple mixture of the active ingredients (1.4.1.) and a second study, were the active ingredients have been inserted into the Alginate based film composition (1.4.2.). Both studies were

carried out over a period of 7-8 days to see the development and interactions between *B. subtilis* and the other components. The tests were performed in duplicate, in two separate experimental runs for safety data readings.

The active ingredients mix

The experiment started by taking in consideration that the producer of *B. subtilis* pure culture declared on the TDS and CoA for this microorganism: $>10^{11}$ CFU (viable spores) of *B. subtilis* /1g. The calculated amount of BS to start for each tube was $0.25x10^{9}$ CFU which was important for the viability after 8 days. Into four sterile tubes labeled: M, P1, P2, P3, a quantity of 10 ml Luria Bertani broth was evenly distributed. In each tube were, aseptically added, at the laminar air flow hood, the active ingredients in the amount and combinations mentioned in (*Table 1*). All tubes were fully stirred using a shaker for a complete homogenization of the aliquot and were maintained throughout the experiment at 4°C.

Daily, for each sample, we made tenfold serial dilution until 10^{-9}, of which we inoculated each 1 mL in sterile Petri dishes on LBA medium in order to count the colony forming units. The plates were incubated at 37°C (Sanyo Incubator MIR-153) for 24-48h. The *B. subtilis* living cells were daily counted in plates which contain 30 to 300 colonies over a period of 7-8 days, according to EN ISO 7218.

Table 1

Sample preparation system for active ingredients. (Amounts for 100 ml tube sample solution)

Sample	*Probisis*®	YE(g)	OE(g)
M	0.25g	-	-
P1	0.25g	-	1g
P2	0.25g	1g	-
P3	0.25g	1g	1g

Alginate based films with active ingredients

The Al base film 1 and 2 recipe is illustrated bellow (*Table 2*). All measurements and equipment were done in aseptic conditions. The active ingredients were integrated into the film forming solutions following the procedure. Film forming gels were prepared according to the method of (Zactiti, Kieckbusch, 2006); were cross-linking of the polymeric structure with calcium ions considerably reduces the solubility in water of alginate based films, as well as the flexibility. Plasticizer (glycerol) is added to these films in order to obtain a reduction in brittleness and increase in flexibility, toughness traction and impact resistance.

Table 2

Film recipe 1 and film recipe 2 with incorporated active ingredients for 100 ml solution

Film Recipe 1	*Probisis*®	YE (g)	OE (g)
1A: Al + KG + CMC	0.5g	0.5g	0.5g
1B: Al + KG + CMC	0.625g	1g	1g
1C: Al + KG + CMC	0.75g	1.5g	1.5g
Film Recipe 2			
2A:Al+ KG+ CMC+ RC	0.5g	0.5g	0.5g
2B:Al+ KG+ CMC+ RC	0.625g	1g	1g
2C:Al+ KG+ CMC+ RC	0.75g	1.5g	1.5g

The experiment started from two optimized simple film recipes with different compositions: **(1)** Al [4.5g] + KG [0.75g] + CMC [0.75g] = 6 g and **(2)** Al [4.11g] + KG [0.63g] + CMC [0.63g] + RC [0.63g] = 6 g. Both compositions were mixed individually in 400 ml double distillate (DD) water under constant stirring until complete solubilization. Then 3.6 g of plasticizer was added to the solution and continued stirring. At this point the three active ingredients were added into the film forming solution after measuring the correct amount which must be introduced. Films were then obtained by casting technique when 5 g of gel was measured into a square Plexiglas pan (area = 225 cm^2) and outspread on the whole surface. Slowly a 10 ml solution ($CaCl_2$, 7% concentration) was added on the Plexiglas pan margins with a dropper; moment when the alginate cross-linking took place and the film was obtained. Each film was then peeled off and put onto Petri dish to dry. For the films containing CMC and KG we also used an ultrasonic water bath were we applied oscillations to eliminate the air bubbles formed while stirring for gel formation. The quantitative microbiological analysis of each Al based film used the (AATCC test method 100-2004, 1961), method originally designed for determining antimicrobial activity of textile materials, and further adapted for edible films.

Each film was cut in a 5 cm diameter disk shape with a sterile scalpel and placed into a sterile Flask disk. The flask disks were incubated at 37°C using a Sanyo equipment. Then 99.0 ml of DD water was aseptically added to each flask at different time frames 24, 48, 72, 96 h. The content was then transferred to a 400 ml homogenizing bag (300x180 mm) and blended in a bag homogenizer for 1 minute. Afterwards dilutions were made of the homogenate in DD water and plated onto Agar plates for the bacteria. The plates were incubated as described above.

Enumeration of colonies was performed, and inhibition of microorganism growth was expressed as reduction of cell number using log (N/N_0)- where N is the viable cell number at a given time and N_0 is its counterpart at time zero (Fernandes J.C. *et al*, 2008). The test was performed in duplicate, for safety reasons.

Design of experiments (DoE)

Using DoE, makes it possible to find one or several best fit solutions for a particular problem. We can accomplish this by using mathematical models to predict the outcome of an infinite number of combinations of the three samples selected. For this we used a Mixture Design I-Optimal Quadratic model with the help of State Ease Trial program. The aim was to find an optimum solution mix for the 3 active ingredients used in order to standardize and create an efficient protection layer. In this case, DoE laid out a „recipe sheet" with a total of 16 blends, of which one was replicated for estimation of pure error.

For the **Components**, the following constraints were respected:

- For the major components (OE and YE) the range was between 0.5-1.5 units.
- For the BS the range was 0.5-0.75 units.
- All the components must sum 3.5 units: A+B+C=3.5.

For the **Response**, the following data were introduced:

- Shelf life :7-8 days
- Color difference (ΔE): 93.82-95.00
- Viability of BS cells: $9.85E^{7}$-$1.75E^{9}$

RESULTS AND DISCUSSIONS

Antioxidant potential of the active ingredients

The antioxidant activity of YE and OE was measured using a distinctive assay: a quantitative method based on DPPH activity. As we concluded the antioxidant activity of YE has a significant value of 33.33%; compared to the OE which has a value of 21.85%. These two ingredients thus they are inserted into the film matrix for their antioxidant and natural preservative effect they assure a synergistic joint venture in protecting the film layer from the pro-oxidative external and internal factors. The EC_{50} value of the YE and OE have a value of 0.88 and 0.60 mg/ml in this case, which represent optimum values for these ingredients. Similar values have been stated by (Huang Z. *et al*, 2009).

Film characterization

Alginate based films color is usually neutral; they are transparent thin layers of polysaccharides used as packaging material to protect the intrinsic value of the food product.

Keeping in mind that we added *B. subtilis*, OE and YE in order to increase the functionality and value of the package, the color of these films also changed: simple films inoculated only with BS are thin (0.08 mm), transparent and flexible; films inoculated with BS and OE are thin (0.09 mm), open brown in color and flexible; films inoculated with BS and YE are medium thin (0.11 mm, Figure 1.a.), yellowish and medium flexible; films inoculated with BS+ OE+ YE are: medium thin (0.12 mm), yellowish-brown and medium flexible. In the film section captured with SEM technology we can observe the different polymer layers which incorporate the bioactive components. The film surface is irregular and rough in aspect with small wrinkles.

The biofilm has a uniform, compact, dense structure. From the interactions of the chemical elements, salts result which crystallize under cubic form with dimensions between 2.47-3.47 µm Figure 1. b. Sometimes these crystals stick together and form uneven cross structures like in Figure 1. c. In the network matrix we can observe immobilized the *B. subtilis* culture cells.

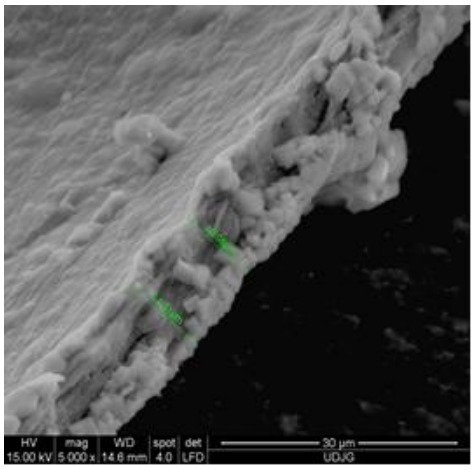

Figure 1.a **SEM images of edible biofilm section**

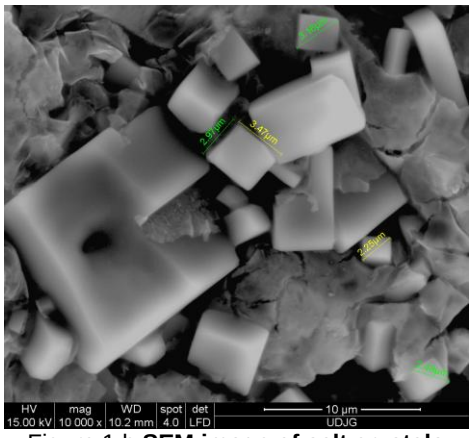

Figure 1.b **SEM image of salt crystals**

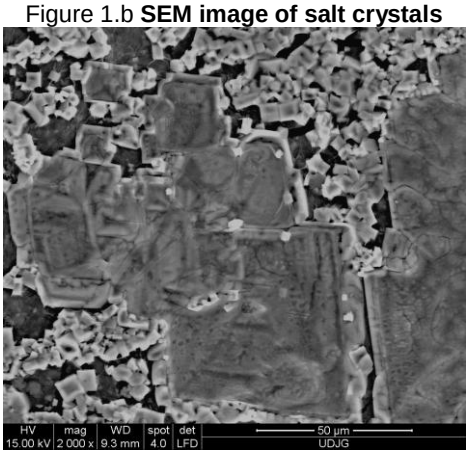

Figure 1.c **SEM image of structure colonies**

1.1. Microbiological analysis

Microbial growth in food is complex. It is governed by genetic, biochemical and environmental factors. The change in bacterial number over time is represented by growth curves showing the lag, exponential, stationary, and death phases of the population. The growth curves

can be plotted as the number of cells (CFU /mL) on a logarithmic scale or $\log_{10}$ CFU /mL versus time. Growth curves graphs represent the state of microbial population rather than individual microbes. Thus, both the lag phase and stationary phase of growth represent periods when the growth rate equals death rate to produce no net change in cell numbers. Table 3 illustrates the resistance of several microorganisms to temperature.

Table 3

Heat resistance of spore-formers of importance in foods (Setlow and Johnson, 2001)

Type of spore	Approx D_{100} (min)
Spores of public health significance	
Group I *Clostridium botulinum* types A and B	7-30
C. botulinum type E	0.01
Bacillus cereus	3-200
Clostridium perfringens	0.3-18
Mesophilic aerobes	
Bacillus subtilis	7-70
Bacillus licheniformis	13.5
Thermophilic aerobes	
Geobacillus stearothermophilus	100-1,600
Bacillus coagulans	20-300
Mesophilic anaerobes	
Clostridium sporogenes	80-100
Thermophilic anaerobes	
Desulfotomaculum nigrificans	<480
Clostridium thermosaccharolyticum	400

B. subtilis strain is present in soil and grass, non-pathogen, used for health benefits, ubiquitous and easy to isolate, resistant in spore form to extreme external factors: temperature, pressure, pH. The *B. subtilis* colonies are generally 3-8 mm in diameter, round, opaque, cream color. Two types of colonies might be observed either small colonies (2mm diameter), white pale with smooth surface or bigger colonies (3-5 mm diameter) with uneven surface wrinkled.

B. subtilis are rod shape bacteria that are Gram positive (Perez A.R. *et al*, 2000). They are about 4-10 μm long and 0.25–1.0 μm in diameter, with a cell volume of about 4.6 fL at stationary phase (Yu Allen Chi-Shing *et al*, 2013). The cell wall is a rigid structure outside the cell. It is composed of peptidoglycan which is a polymer of sugars and amino acids. Figure 2 depicts the microscopic aspect of *B. subtilis* strain that we used in our experiments above.

The data reveals that practically every mix performs well for cell multiplication. Of course there are some small variations between the studied samples, but in acceptable limits.

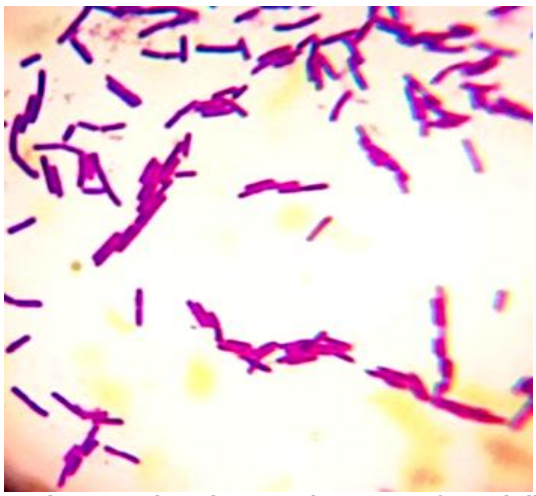

Figure 2. The microscopic aspect of *B subtilis* strain stained with Fuschin, 100x objective (oil)

For the control sample (M) were we have inoculated the plate only with *B. subtilis* the viability over more than 7 days is reaching a level of $7.2x10^8$ CFU/g, which is much considering that there is no other interactions. This simple solution presents also a natural lantibiotic, subtilin, which is produced by *B. subtilis*. This bacteriocin contains five lanthionine rings and has a conformation similar to nisin. For P1 sample which represents a mixture of BS and OE the results are lean in the sense that we obtained a population of only $9.85x10^7$ CFU/g. This can be logically explained due to the onion extract which can be characterized as one of the best plant antimicrobials, going hand in hand with garlic.

The onion extract inhibits *B. subtilis* and many other microorganism developments due to the bacteriocins contained who target the cell membrane of sensitive bacteria. They disrupt the membrane by making pores.This increases permeability to small compounds, causing a rapid efflux of preaccumulated ions, amino acids, and, in some cases, adenosine 5-triphosphate (ATP) molecules. A major reaction occurs while using onion extracts, forming thiopropanal-S-oxide.

Onion extract contains also antimicrobial phenolic compounds, protocathechuic acid and

cathechol (Montville T.J. *et al*, 2012). Therefore, their modes of action are probably related to those of other phenolic compounds which interfere with the membrane function of the microorganism. For P2 sample we have a mixture of BS and YE which manage to provide outstanding results in CFU value.

The cell growth of *B.subtillis* incorporated in Al based films were plotted in Figure 4. For film recipe 1, Al+ KG+ CMC were used as base film materials in which we incorporated the active ingredients. Sample 1A shows a poor result in CFU/g value reaching the level of $8.4x10^8$ after 8

days. This result can occur due to the fact that sample 1A had been inoculated with the smallest amount of BS. For samples 1B and 1C the results

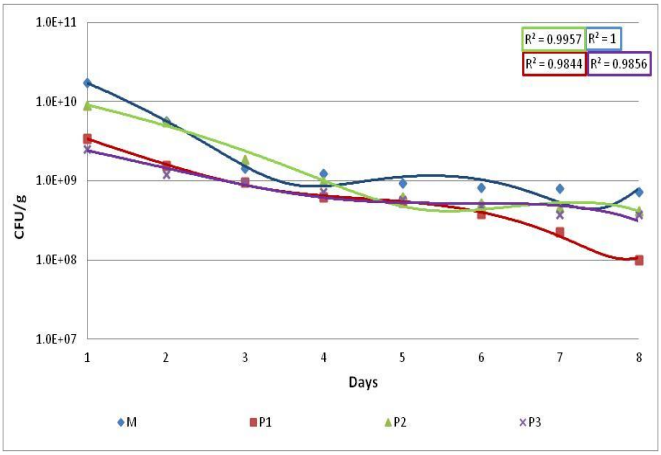

Figure 3 **The influence of active elements from OE and YE over probiotic viability of *B. subtilis* in 8 days preservation at 4°C**

Here we reach a maximum of $4.15x10^8$ CFU/g due to YE which potentiates the BS development due to the high amount of nitrogen which is necessary in microbial metabolism. This YE has in composition a minimum of 55% protein and is rich in glutathione. GSH is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides and heavy metals (Pompella A. *et al*, 2003). Another big advantage is that this YE obtained from *Saccharomices cerevisae* provides a taste and color benefit: specific Kokumi taste and yellowish color due to the sulfur rich content.

For P3 sample which represents a mixture of BS+ YE+ OE we have achieved an optimum result in the growth of the microbial population. This represents a balance point were OE and YE somehow act synergicaly and potentiate the development of BS which reaches a value of $3.75x10^8$ CFU/g.

For film recipe 2; Al+ KG +CMC+ RC were used as base film materials in which we incorporated the active ingredients. Samples 2B and 2C show similar values of $1.3\text{-}1.4x10^9$ CFU/g after 8 days which represent interesting data for the microorganism encapsulated into the film matrix. For sample 2C we get the highest result of $1.8x10^9$ CFU/g of BS which is normal to the fact that 2C has been inoculated with the higher concentration of the microorganism.

Microbiological aspect of edible films

To determine the quality of alginate based composite films, we used the SEM equipment Quanta 200 in order to observe the morphology and dispersion of the polymers and the active ingredients and bacterial cells into sheets. Figure 5. illustrates the images of alginate based films

are promising reaching CFU values of $1.1\text{-}1.3x10^9$ CFU/g. These similar results are usual for the active ingredients inside.

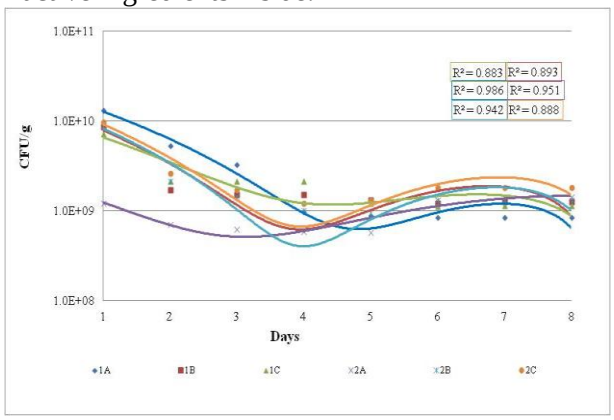

Figure 4 ***B. subtilis* cell growth in Alginate based films**

from different perspectives. The *B. subtilis* is a rod shape bacteria that typically forms small clumps, short chains or single cells. During biofilm development, *B. subtilis* switches from being flagellated, motile single cells to growing in long chains of nonmotile cells that form parallel bundles (Branda SS. *et al*, 2006). Once this matrix is produced, the community develops a high degree of spatiotemporal organization culminating with sporulation occurring preferentially at the tips of aerial structures.

Images (1A, 1B and 1C) provide a clear perspective over the surface of the Al based films which incorporated the active ingredients and how the microorganism developed by creating snow flake small colonies (1A) or overlap each other in big colonies with cross-shape and irregular surface.

These are uniformly distributed in the film structure (1B and 1C). Images (1A', 1B' and 1C') are taken with the magnitude of 5000x and illustrate the *B. subtilis* population density which is homogeneously distributed. (1A') is more sporadically in numbers while (1B') and (1C') is very consistent; this being directly correlated with the amount of *B. subtilis* inoculated into the recipe.

For images (2A, 2B and 2C) the situation is similar like to film recipe 1 described above. (2A') illustrates at 10.000x, a heterogeneous display of the cells into the polysaccharide film matrix. In this case a thin layer of gel surrounds the rod shape walls of the grown culture. Image (2B') shows a great abundance of crystal structures with cuboids shapes and different dimensions. We assume that these crystals represent salt residues which are used to standardize the polysaccharides for the film

matrix. For film sample (2C') images provide good details about the agglomeration and stickiness of the pure culture. In this case the reading has been realized with 2000x magnification.

Design of experiments (DoE)

DoE provides five possibilities for a "Goal" to construct desirability indices (d_i): none (to disregard any given response), maximize, minimize, target, in range (simple constraint) and equal to (components only). Desirability's range from zero to one for any given response. The program combines individual desirability's into a single number and then searches for the greatest overall desirability. A value of one represents the ideal case. A zero indicates that one or more responses fall outside desirable limits. DoE uses an optimization method developed by Derringer and Suich, described by (Myers R.H. *et al*, 2009).

For the components of the mix there are no changes and every "Goal" is set to "In range" option.

For the response we set the "Goal" to **Target** and maximum importance for the following

-Shelf life, target 8 days;

-Color difference, to in range;

-Viability of BS cells, target $1x10^9$ cfu/g.

A desirability formula was used to determine the optimized solution were a **92,60%** desirability was a little better than the screening experiment. Also the active mix performance was actually much closer to the benchmark within the margin of error. For the other three Optimized contour graphs, the Design is strictly correlated between: time, ph value and growth rate factors.

This happens due to a direct connection between these elements and the development of *Bacillus subtilis.* For Shelf life contour graph the program flagged a prediction of 7.9 days. If we increase the targeted value to a value of 9 or more days we risk to fall out of the targeted settings and influence the experiment.

At a second look in the Shelf Life the chances of BS evolution, increase if we aim for 9 or more days, which is normal for this bacteria. For the Color difference contour graph the value predicted by the program is around 93.84 which is important because the films have a more brownish color. Here we can observe a color gap between the two active ingredients onion extract and yeast extract. They are situated in opposite corners. If the OE provides a clear brownish color to the films on the other side the YE provides a more yellowish aspect. In the Viability contour graph an increase in cfu units is visible if we increase the amount of BS and EC. These 2 ingredients repeal each other. This is why the whole picture must be studied and not base our results only on a contour graph. Mixture design provides an understanding of interactions between all of the ingredients with respect to the desired properties so that future formulation development becomes less reliant on trial and error.

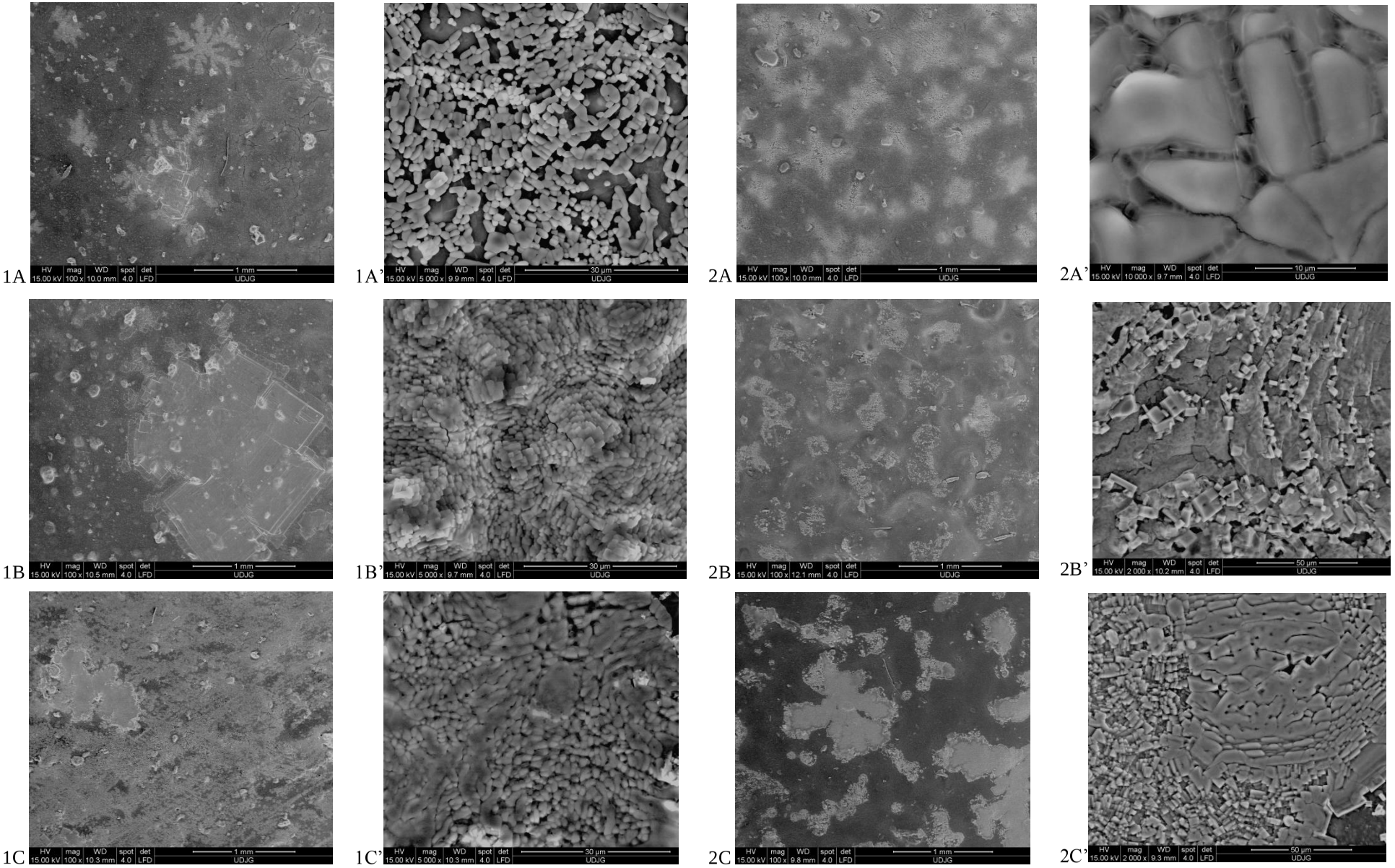

Figure 5 **Electron microscope images for recipe 1 and recipe 2**

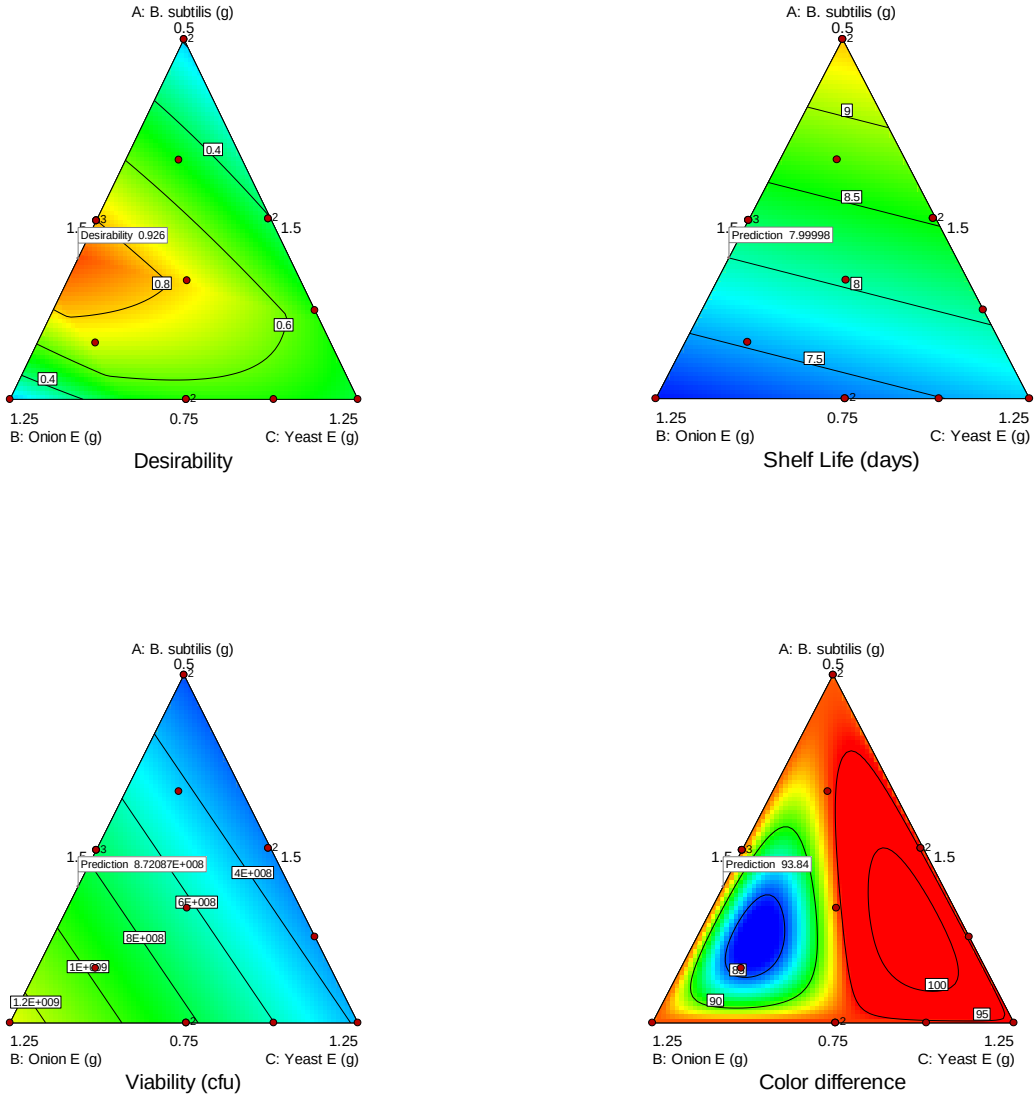

Figure 6 **Desirability, Shelf life, Color difference and Viability of BS cells –Optimized contour graphs**

CONCLUSIONS

This study demonstrates that obtaining Al based films and incorporating distinct active ingredients with unique properties provide a „clean" solution and a future reference to healthy packaging materials. An interesting approach was made in the sense that we could study the interactions between YE, OE and the development of *B. subtilis.* Most of the *B. subtilis* biofilm data comes from studies on the development of complex, wrinkled colonies and from the development of pellicles at an air-liquid interface.

Through SEM analysis we could gather information about how the polysaccharide matrix incorporates the cells of probiotic culture and the conformation which this takes after the sporulation process during the preservation of the In both studies, we focused on the time frame of minimum 8 days to examine the development and resistance of *B. subtilis.*

In both cases the results are promising. For the single active ingredients used the best result came from sample P_2 (*B. subtilis* + yeast extract) were the number of CFU reached a maximum of $4.15x10^8$. In the second case for the Al based films the best solution was established with Al + CMC + RC + KG and a mixture of *B. subtilis* + yeast extract + onion extract. Here we had a number of $1.8x10^9$ CFU/g.

Last but not least we can optimize our results and mixtures by using DoE which combines statistic and mathematical analysis in obtaining an optimum recipe for our film. All this data represent a big advantage for future development of active packaging for fresh products.

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