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Obtention of New Edible Biofilms from Water Kefir Grains in Comparison with Conventional Biofilms from Taro (*Colocasia esculenta*) and Cassava (*Manihot esculenta*) Starch

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Abstract: Microorganism biomass is a sustainable and innovative source of biopolymers, such as proteins and polysaccharides, that is suitable for the development of biodegradable films. The aim of this research was to evaluate the synthesis, morphology, rheology, and morphological and mechanical properties on the production of edible biofilms based on water kefir grains, and compare them with edible films based on thermoplastic compounds from starch (TPS) obtained from taro (*Colocasia esculenta*) and cassava (*Manihot esculenta*). Edible biofilms were prepared in solution with 30% wt/wt glycerol relative to starch mass and kefir grain biofilms using the casting method. A stationary rheological analysis was performed on the film-forming suspensions of kefir, taro starch, and cassava starch. Once the films were obtained, a physicochemical and morphological characterization was carried out. Results of the characterization showed the following main aspects: The results indicated an increase in biomass production using muscovado and pineapple peel. The film-forming suspensions had a dilating behavior; however, the results obtained not only show the viscoelastic behavior but also the elastic limit (σ_0), which varied from 0.077 to 0.059 Pa for suspensions of water kefir grains and from 0.077 to 0.072 Pa for starch suspensions. These elastic limit variations can be defined as the minimum shear stress required to start the flow, and all these rheological data were adjusted to the Herschel–Bulkley model; the morphological and mechanical characterization of the films obtained showed homogeneous surfaces with transparency and without cracks; regarding the water activity, values lower than 6 were obtained, which indicates that there will be no growth of any microorganism, and the hardness data showed differences between those obtained from kefir and taro and cassava starch. The similar results of the rheological characterization in the formation of the kefir biofilm and the conventional edible starch films, in addition to the similar results in the water activity below 6 and the hardness, points to an attractive alternative capable of replacing the conventional materials with a mass production of biofilms of probiotic microorganisms. The results also revealed that water kefir grains biomass is a viable and innovative source of biodegradable materials, and these grains can be an alternative to conventional established starch materials.

Keywords: water kefir grains; biobased films; thermoplastic starch; rheology; water activity; hardness

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1. Introduction

In recent years, interest in biodegradable edible materials has increased as an alternative to non-biodegradable petroleum-derived materials and synthetic plastics for reducing its impact on the environment and human health. Nowadays, this has acquired great importance in the development of new materials for substituting, at least partially, the traditional food packaging plastics, which show significant shortcomings in terms of their environmental impact [1]. Environmental problems can thus result from using non-renewable raw materials and accumulation of such non-biodegradable materials [2]. Biodegradable films are used in various fields, such as packaging, storage, and even

medicine, due to the important role they play in the prevention of cardiovascular diseases, cancer and diabetes [3], so that they could replace non-biodegradable plastic. The continuous awareness of scientists towards environmental pollution in recent years and consequently the need for a safe and ecological environment has caused a paradigm shift in the use of biodegradable materials. In industrial fields, the main biofilms used are those produced by plants (pectin, starch) and algae (alginate, carrageenan), animal protein hydrocolloids (gelatin, casein) and some microbial polysaccharides (dextran, xanthan, gelling agent, curdlan) [4]. A new alternative in biofilm production is the use of water kefir grains. Currently, tendency in the packaging industry is focused on developing new materials that are cheaper and more durable than traditional materials [5–7]. In particular, there is a growing interest in renewable and/or biodegradable membranes obtained from film-forming substances such as proteins, polysaccharides and lipids [1,5,6]. These biodegradable films and coatings can be used to coat food surfaces and act as barriers to control the transfer of moisture, oxygen, carbon dioxide, lipids, and ingredients that preserve flavor, thereby maintaining quality and increasing shelf-life of food products [8]. Among other important properties, these films can be used as carriers for functional agents, such as antimicrobials or antioxidants, and to improve food appearance and handling [9].

Water kefir grains are a bacteria/yeasts consortium that live in symbiosis, and the interactions between these different organisms can arise in different ways. Physical associations can occur in loose communities that rely on special signaling molecules (quorum sensing) or symbiotic associations with adhesion factors such as proteins or polysaccharides (biofilm). These associations may be based on different molecular interactions, such as adjustment of the physicochemical environment (pH change) [10]. Film obtained by molding involves the use of at least one film-forming agent (film-forming agent such as polysaccharide or protein), solvent, and plasticizer. Regarding the film matrix formation, a dispersant that contains the biopolymers is prepared and then the solvent is removed (drying method) in order to decrease the separation between the polymers improving thus their interaction. This interaction allows the formation of a macromolecular network leading to a structure in the film [11]. The film properties depend on the structure and chemistry of the polymer chains, the melting conditions, and the presence of plasticizers [1]. Plasticizers are required to improve film integrity and mechanical properties [5,12]. They are usually small molecules that destabilize hydrogen bonds, decrease intermolecular forces, and increase the sterility and mobility of polymer chains [13]. Several studies have reported the use of polysaccharides from different sources to obtain films and coatings [1–18]. However, in recent years, the study of materials obtained from microbial exopolysaccharides has increased [19]. This new biofilm replacement method based on aqueous kefir particles can be compared with conventional starch-based films. Aqueous kefir grains contain dextran, which is a polymer of glucose, composed mainly of straight αD1,6-linked side chains with a small percentage of αD1,3-linked side chains [20]. The content of this polymer is the most important difference with respect to milk kefir, which has a more complex heteropolysaccharide called kefiran [21]. Regardless of this difference, these types of kefirs contain similar groups of microorganisms: lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeast [22,23]. The symbiotic microorganisms are embedded in the polysaccharide matrix forming a complex structure. Several authors have identified different yeast. The objective of this research was to evaluate the preparation, morphology, rheology, and properties of the production of edible biofilms based on water kefir grains and compare them with films based on thermoplastic compounds from starch (TPS) of taro (Colocasia esculenta) and cassava (Manihot esculenta). Water kefir grains have no cost in the market; they are acquired from person to person. It is important to increase biomass production since the more biomass there is, the more biofilms or biomaterials can be formed. There is an interest in the study of materials obtained from microbial exopolysaccharides, and the biofilms of water kefir grains could turn out to be an attractive solution for the substitution of non-biodegradable plastics.

2. Materials and Methods

Figure 1 shows the general methodology of the obtention and characterization of new edible biofilms from water kefir grains and conventional edible films from taro and cassava starch.

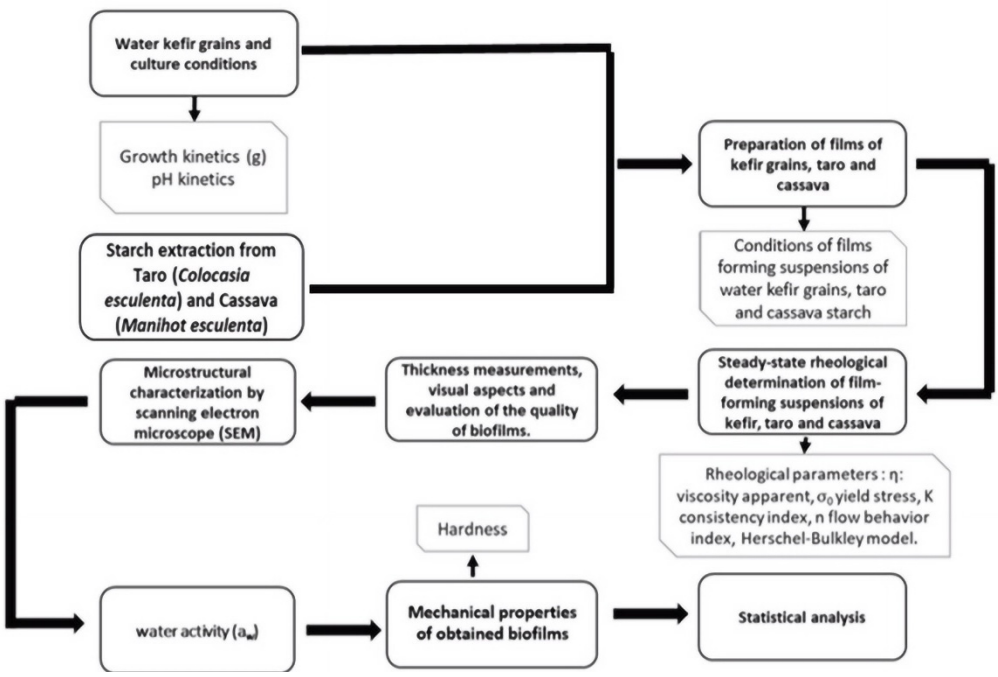

Figure 1. General methodology of the process for obtaining new edible biofilms from water kefir grains in comparison with conventional films from Taro (*Colocasia esculenta*) and Cassava (*Manihot esculenta*) starch.

2.1. Water Kefir Grains and Culture Conditions

Water kefir grains, used as a starter culture, were obtained from a private household in Orizaba, Mexico and stored frozen at −20 °C. These grains were reactivated by consecutive infusions at room temperature in 2 L of water culture medium containing 100 g of kefir and 100 g of muscovado sugar. Medium was changed daily with fresh culture medium to maintain grain viability. Subcultures were repeated several times to increase water kefir biomass. The growth conditions of the water kefir grains were evaluated for 1 L of water and sucrose with pineapple peel on a dry basis for eight days to observe their growth and reproduction.

To evaluate the growth conditions, the following factors were considered: substrate concentration (Sucrose) 25 and 17 °Brix, temperature 25 and 35 °C.

Another very important factor taken into account was variation in the origin of the substrate using muscovado sugar (P25 and P17), refined sugar (A25 and A17), muscovado sugar with pineapple skin (PP25 and PP17) and refined sugar with pineapple skin (AP25 and AP17).

The biomass concentration remained constant in 10 g of water kefir grains and 10 g of dry pineapple peel.

Biomass growth kinetics was determined using the drained weight method [24] with an analytical balance (Ohaus Explorer). pH level was measured with a digital potentiometer (Hanna Instruments HI 2210) during the eight days of fermentation. To prepare the film, the granules were separated from the fermentation product by filtration through a plastic sieve, washed three times with distilled water, and pressed to remove excess water. The dry matter of washed and pressed kefir grains was 0.1 g per g as determined by drying at 105 °C remaining constant in all treatments. The final product obtained was biomass of dry water kefir grains ready to be processed for the biofilm synthesis. Table 1 shows

the experimental design used in this section where the factors were temperature, substrate concentration and source of the substrate, identifying Y_a as the response variable of the growth kinetics (g) and pH at 25 °C and Y_b as the response variable of the growth kinetics (g) and pH at 35 °C.

Table 1. Design of experiments of the culture conditions of the grains of water kefir growth kinetics and pH.

	Temperature °C	**25 °C**		**35 °C**	
	Substrate Concentration (°Brix)	**25 °Brix**	**17 °Brix**	**25 °Brix**	**17 °Brix**
Source of the substrate	Muscovado sugar (P)	Y_a = P25	Y_a = P17	Y_b = P25	Y_b = P17
	Refined sugar (A)	Y_a = A25	Y_a = A17	Y_b = A25	Y_b = A17
	Muscovado sugar with pineapple skin (PP)	Y_a = PP25	Y_a = PP17	Y_b = PP25	Y_b = PP17
	Refined sugar with pineapple skin (AP)	Y_a = AP25	Y_a = AP17	Y_b = AP25	Y_b = AP17

2.2. Starch Extraction from Taro (Colocasia esculenta) and Cassava (Manihot esculenta)

Taro and cassava tubers were collected at the local market "Emiliano Zapata" in Orizaba, Veracruz, Mexico. The raw material (taro and cassava) was washed with water and neutral soap. Once it was washed, taro and cassava were separately handled, peeled, and cut into medium pieces. Later, both tubers were submerged in a 0.03% citric acid solution to prevent enzymatic browning and then the pieces were crushed using an industrial processor.

Starch processing was separately carried out for each sample of taro and cassava. The dry starch was crushed with a mortar and pestle, and sifted through different meshes, 100, 200 and 400 (0.150, 0.075 and 0.038 mm, respectively) to decrease the particle size. Then, the mixtures were allowed to settle for 24 h and dried in a tray dryer at 45 °C for 8 h for obtaining a finer starch. The dry starch was stored in hermetically sealed plastic bags until further use [25]. A similar method was used to obtain starch from cassava where Starch purity varied from 95 to 98% [26]. In the same way, this method was used for the extraction of taro starch and its purity was 98% [27], i.e., these tubers in their composition are mainly formed by starch than by other components.

2.3. Preparation of Kefir Grains, Taro and Cassava Edible Films

Washed and pressed kefir grains were used to prepare a 3% dry weight dispersant film of water kefir grains. The dispersion was first homogenized at 15,000 rpm for 5 min using an Ultraturrax T25 (IKA® ULTRA-TURRAX) disperser tools of stainless steel. It was then homogenized by ultrasound (Rotary homogenizer-EW-04711 series-Cole-Parmer-ultrasonic/sample preparation/benchtop) at 80 W for 15 min, followed by heat treatment in a water bath at 90 °C for a period of 20 min. A second ultrasonic homogenizer was applied with the same conditions as the previous homogenizer. A first homogenization process was required to separate the compact structure of the particle and the cell wall of the remaining microorganisms to produce a fine dispersion. A heat treatment was performed to denature and unfold the film-forming agents and a final homogenization was performed to break the aggregates formed by the heat treatment [16]. Pure glycerol was added to the dispersion in a proportion of 30% of dry matter (d.m.) weight relative to the weight of water kefir grains. After adding the plasticizer, stirring was applied for 15 min. No additional heat treatment was applied to the dispersions after the addition of glycerol. Finally, the dispersants were placed under vacuum for approximately 30 min to remove air bubbles created during treatment. The final pH of the dispersants was 4.5 and this was set with a pH-ORP meter (Hanna Instruments 2211). For pH meter calibration, buffer solutions with pH of 4.01 and 7.00 were used.

To obtain thickness close to 1 mm and 17 g on our films, the scatterer was placed in plastic Petri dishes with a diameter of 8.6 cm. Water evaporation was achieved by casting at

40 °C and 40% relative humidity (r.h.) in a ventilated oven, until the residual water content of the film was between 10 and 15%.

Subsequently, suspensions of THPS (thermopolysaccharides) of taro and cassava starch were prepared using an unifactorial design where the concentration of starch was varied at 4.5 and 5.5 g levels in 100 mL of water using glycerol as a plasticizer at 30% wt/wt of the weight of the starch at 25 °C [28].

The films were then stored at 22 °C and 43% r.h. Finally, as required by the experiment, the membranes were equilibrated in desiccators using P_2O_5, LiCl, $KC_2H_3C_2$, $MgCl_2$, K_2CO_3, $NaNO_2$, KCl and NaCl saturate solutions to create the conditions of 0, 11, 33, 35, 57, 65, 75 and 90% r.h., respectively. The dry atmosphere was obtained using silica gel.

To better illustrate the film formation conditions, Table 2 shows the factors used for each edible film treatment of water kefir grains, taro, and cassava in which only the concentration of starch for taro and cassava varied.

Table 2. Main conditions for the preparation of films (Concentration).

Water kefir grains 3% wt/wt
Taro 4.5 and 5.5 g
Casava 4.5 and 5.5 g

The plasticizer was kept constant for all samples with a ratio of 30% by weight of the dry matter of kefir, taro and cassava starch.

The molding method was used for the formation of films.

2.4. Steady-State Rheological Determination of Film-Forming Suspensions of Kefir, Taro and Cassava

The rheological analysis of the film-forming suspensions SFP (SFP taro and SFP cassava) was carried out as follows: the rheological behavior was analyzed by shear tests in steady state, using an Anton Paar model MCR301 rheometer at a shear rate of 0.1 to 1000 s^{-1}. Shear experiments were performed at two temperatures (25 and 35 °C), using a stirring geometry (ST22-4V-40-SN10120) and a Peltier cylinder (C-PTD200-SN80123149) in order to obtain the rheograms and shear parameters [29]. Rheological tests were also performed in rotational state to check the type of behavior of the suspension. Moreover, the Herschel–Bulkley (HB) model fitted on the data from all experiments.

$$\sigma_{HB} = \sigma_{0_{HB}} + K_{HB}\dot{\gamma}^{n}, \quad (1)$$

where $\sigma_{0_{HB}}$ is the yield stress (Pa), which is regarded to be an indication of the specific value of stress exerted on the SFP when it begins to flow [30], K_{HB} is consistency index ($Pa{\cdot}s^n$), $\dot{\gamma}$ is the shear rate (s^{-1}) and n is the rheological behavior index (dimensionless). The interpretation of the value of this last parameter has great importance for the determination of the behavior, where n = 1 indicates a Newtonian behavior, n < 1 indicates a pseudoplastic behavior and n > 1 a dilatant behavior [31].

2.5. Thickness Measurements, Visual Aspects and Evaluation of the Quality of Edible Films

Before proceeding to the determination of all the edible films, the thickness was measured using a Mitutoyo model IP65 Coolant Proof micrometer, with an interval of 0–1 inches. The measurement was made at 10 different points of each edible film, obtaining an average value of the final thickness [32].

The visual appearance of the film was captured by photographs. The quality of the films obtained was evaluated taking into account the aspects of feasibility, uniformity and appearance [33]. This evaluation was performed with three independent replicates. For all the tests described below, only the films with the best visual appearance and quality were used.

2.6. Microstructural Characterization by Scanning Electron Microscope (SEM)

The surfaces of the films were analyzed using a SEM-Jeol model JSM6510 scanning electron microscope. Sections were obtained by cutting with a sharp blade at room temperature. For a better visualization under the microscope, images of the faces were obtained (500, 1000 and 2500× magnification) and the membranes of the water kefir, *Colocasia Esculenta* and *Manihot Esculenta* were analyzed.

2.7. Water Activity (a_w)

The water activity (dimensionless) of the edible films placed in the saturated solutions of the salts was measured with an a_w meter (AQUALAB 4TE) according to the method 978.18 analysis of the de la AOAC (1980) [34]. The measurement was made at the beginning and when the sample reached a constant weight. The samples analyzed were taro, cassava, and kefir grain films. The edible films were brought to a minimum humidity value and placed them in a desiccator with phosphorus pentoxide (P_20_5) for approximately 90 days until a constant weight was obtained. The dry samples were located in stainless steel mesh trays, which in turn were placed in the sample holders that contained the saturated solution of the salts (Table 3) at 25 °C simulating atmospheres with different a_w to evaluate if the edible films are microbiologically stable.

Table 3. Water activities of saturated solutions.

Solution	Name	a_w
		25 °C
P_2O_5	Phosphorus pentoxide	0.000
LiCI	Lithium chloride	0.115
CH_3 COOK	Potassium acetate	0.226
$MgCl_2$	Magnesium chloride	0.327
K_2CO_3	Potassium carbonate	0.438
Mg $(NO_3)_2$	Magnesium nitrate	0.528
$NaNO_2$	Nitrate of sodium	0.577
NaCI	Sodium chloride	0.765

2.8. Mechanical Properties of the Edible Films Obtained

The Shore A hardness test (also called a durometer hardness test) is used when the material is too soft to measure with a Rockwell test, therefore, for very soft materials such as rubber or TPE the Shore A test is recommended.

Shore A hardness is a standardized test that consists of measuring the penetration depth of a specific indenter. The test methods used to measure Shore A hardness are ASTM D2240 and ISO 868 [35]. The hardness value is determined by the penetration of the foot of the durometer indenter into the sample. Shore Hardness measurements are dimensionless; they range from 0 to 100, where the higher number represents the harder material. The resulting depth depends on material hardness and its viscoelasticity.

2.9. Statistical Analysis

Statistical data was analyzed using KaleidaGraph. ANOVA was applied to analysis of variances of the properties with a confidence interval level of 95% and Tukey–Kramer test was carried out to assess if there could exist a significant difference in the mean results of the experiments.

3. Results and Discussions

3.1. Biomass Growth of Water Kefir Grains and pH Kinetics

The biomass curves of the different samples are shown in Figure 2a,b. The codes used in the Figures 2 and 3 are found in the methodology Section 2.1. It was observed that the greatest growth occurs with the muscovado sugar-pineapple substrate at 35 °C and 25 °Brix

(PP25). Muscovado sugar turned out to be the best substrate for kefir grains [24] but it was observed that pineapple peel accelerated and increased microbial growth in curves PP25, AP25 and AP17 (muscovado sugar-pineapple at 25 °Brix, refined sugar-pineapple at 25 °Brix and refined sugar-pineapple at 17 °Brix) and the best results were observed at 25 and 35 °C. Comparing the samples with pineapple and without pineapple, the PP25 and AP25 samples were the fastest growing. It is also observed that in both cases, the maximum growth occurred on the third day, and from the fourth day biomass inhibition was perceived. These results have a high potential since this fermentation serves as a probiotic drink [36] and increases the biomass for the generation of films.

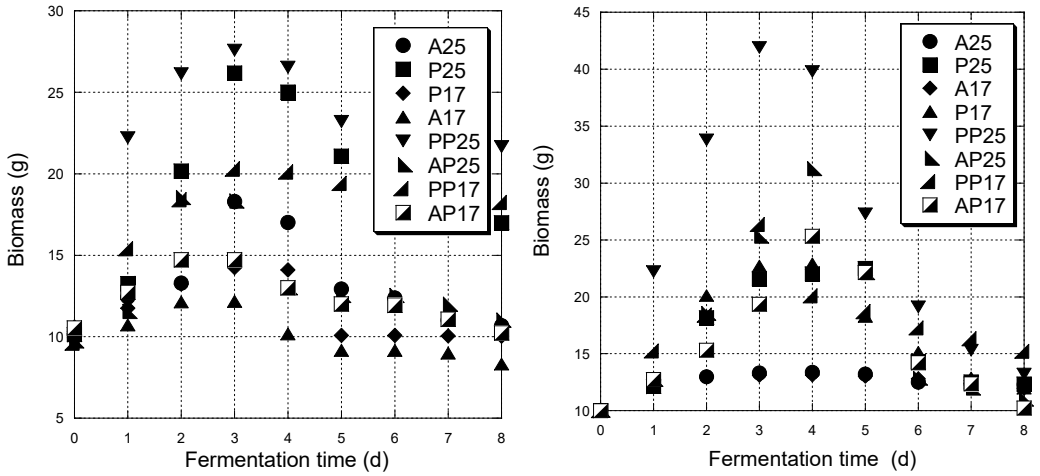

Figure 2. Growth kinetics of water kefir grains (**a**) at 25 °C and (**b**) at 35 °C.

The pH kinetics are shown in Figure 3a,b. During fermentation, the gain of H+ ions occurred, which indicates that the sample was acidified, with an average pH of 3 on day 4, and therefore, biomass growth was affected. The metabolic activity of microorganisms and the production of acids could be inhibitory in the development of some microorganisms [24].

Some authors have reported the decrease in pH in sugary substrates when they are fermented by the consortium of microorganisms in the kefir grains [37] using 10 g/L of different unrefined sugars (demerara, brown and molasses) as kefir fermentation substrate (0.6% inoculum), in which they analyzed the decrease of the pH for a period of 7 days adjusting the initial pH to 5 with 0.1M HCl. These authors determined the minimum pH of 3.3 for demerara sugar, 3.4 for brown sugar, and 3.5 for molasses after 48 h of incubation at 25 °C. In this investigation, minimum pH levels of 3 were found in longer periods of time (4 days) at 25 and 35 °C in which muscovado sugar inoculated with 10 g of kefir grains was used as a substrate. These results allowed us to conclude that the muscovado sugar with pineapple peel addition in dry basis is a suitable medium for the cultivation of kefir grains prolonging its own inhibition.

Several articles were studied and, according to a study that discusses the yield in the growth of kefir grains [38], the pH results coincide with those obtained in this investigation. However, the yield was not affected by the levels of pH but due to other factors such as the origin of the kefir grain, the substrate, and the treatment that was given during growth.

The growth of the microorganisms present in the kefir grain is directly related to the increase in biomass, since by increasing the viability of the microorganisms present in the grain with the consumption of substrate, the yield increases as well [39].

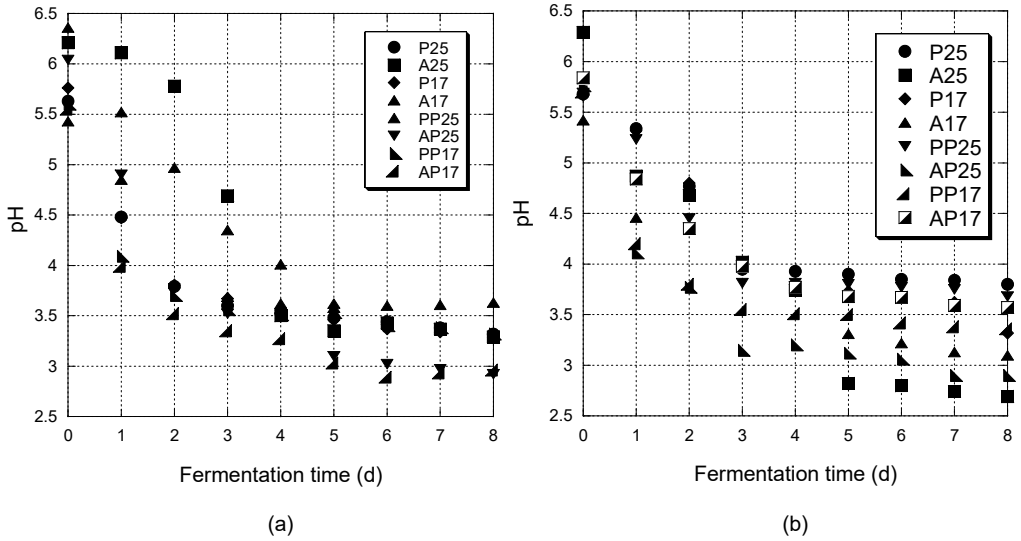

Figure 3. pH kinetics (**a**) at 25 °C and (**b**) at 35 °C.

3.2. Steady State Rheological Determination of the SFP Film-Forming Suspensions

If the viscosity increases according to the shear rate, the result is a dilating fluid (behavior that tends to form a solid) edible behavior of the film-forming suspensions of both kefir and starch. This type of fluid is characterized by an increase in viscosity that is called apparent viscosity of the fluid when the rheological behavior index (n) is greater than unity, and in this study the flow behavior index for all SFP was 1.8 on average, which confirms dilatancy. Concentrated suspensions exhibit this coarsening or thickening behavior at high shear rates, but the occurrence and importance of dilatancy effects are highly dependent on the volume fraction, particle size distribution, and viscosity of the suspension fluid. The shape of the particles is also very important, and the anisotropy (variation of the general properties of matter according to the direction in which they are examined) of the particles tends to produce thickening effects at a low shear rate and for a fraction of smaller solids. The increase in viscosity has been attributed to a transition from a stratified two-dimensional array of particles to a three-dimensional (random) shape. The layered arrangement of the particles allows the suspension to flow easily, as each layer moves without obstruction by other particles [27]. If, on the contrary, the viscosity decreases according to the shear rate, it is a pseudoplastic fluid (behavior that tends to form low-viscosity fluids) behavior that has been demonstrated for film-forming suspensions of *E. coli* [40,41]. This type of fluid is defined as the decrease in viscosity with the increase in shear rate and the rheological behavior index (n) is less than unity. This behavior is found with concentrated suspensions in Newtonian fluids when the solids have particle-particle interactions. As the flow rate increases, the bonds between particles are broken, the mobility d of the particles increases, and the viscosity of the suspension decreases [27]. Steady-state water kefir suspension rheograms are shown in Figure 4a,b. The SFPs at 25 and 35 °C presented a non-Newtonian behavior. Its flow behavior index greater than one (n > 1), confirmed dilatancy (shear thickening behavior) [29]. This behavior is similar to that reported in the production of bioethanol for mash, fermented mash and impure bioethanol [27], and its flow curves fit well (R^2 = 0.99) to the Herschel–Bulkley model. It was observed that, for samples of muscovado sugar and samples containing pineapple peel, the dilatancy was higher and σ_0 at 35 °C turned out to be lower compared to those at 25 °C, which indicates that less effort is required to flow. All the parameters of the Herschel–Bulkley model Equation (1) are shown in Table 4. All the tests showed satisfactory confidence (95% < θ > 99%). Figure 5 shows the rotational rheograms of the taro starch suspensions where a non-Newtonian behavior with dilating characteristics is observed, that is to say, as the shear rate increases, the viscosity also increase. Table 5 shows the rheological parameters of the Herschel–Bulkley (HB) model, highlighting the flow behavior

that, being greater than 1, demonstrates the dilatant behavior of the taro suspension. The codes used in Figure 4 are found in the methodology Section 2.1 and the codes used in Figure 5 are found in the methodology Section 2.4.

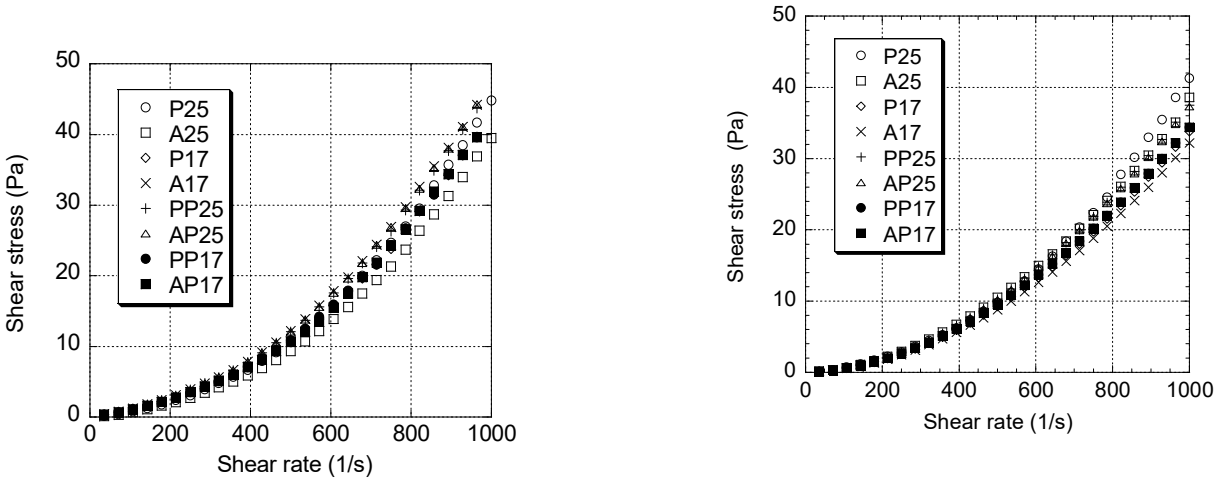

Figure 4. Water kefir SFP samples behavior (**a**) at 25 °C and (**b**) at 35°.

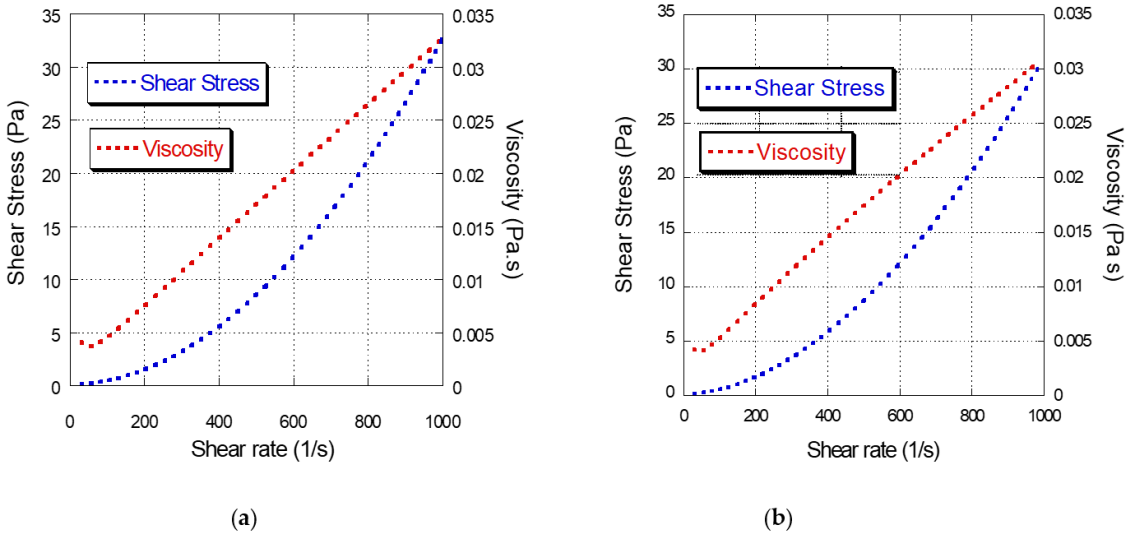

Figure 5. Rheogram of taro SFP at 25 °C at the two levels of starch concentration (**a**) 4.5 g and (**b**) 5.5 g.

Figure 6 shows the rheograms in the rotational regime of cassava suspensions where a non-Newtonian behavior with dilating characteristics is observed, that is, as the shear rate increases, the viscosity increases. Table 6 shows the rheological parameters of the Herschel–Bulkley (HB) model, highlighting the flow behavior that, being greater than 1, demonstrates the dilating behavior of the cassava suspension.

Table 4. Parameters of the steady state analysis of the Herschel–Bulkley model for water kefir (Wk) SFP.

Wk-SFP Samples	Temperature [°C]	Experimental Model $\sigma_{HB}=\sigma_{0_{HB}}+K_{HB}\dot{\gamma}^{n}$	R^2
P25	25	$\sigma = 0.077 + 10.4 \times 10^{-5}\dot{\gamma}^{1.865}$	0.991
	35	$\sigma = 0.067 + 10.4 \times 10^{-5}\dot{\gamma}^{1.863}$	0.991

Table 4. *Cont.*

Wk-SFP Samples	Temperature [°C]	Experimental Model $\sigma_{HB}=\sigma_{0_{HB}}+K_{HB}\dot{\gamma}^{n}$	R^2
A25	25	$\sigma = 0.059 + 9.4 \times 10^{-5}\dot{\gamma}^{1.861}$	0.990
	35	$\sigma = 0.059 + 9.4 \times 10^{-5}\dot{\gamma}^{1.850}$	0.990
P17	25	$\sigma = 0.056 + 9.7 \times 10^{-5}\dot{\gamma}^{1.856}$	0.991
	35	$\sigma = 0.064 + 9.8 \times 10^{-5}\dot{\gamma}^{1.880}$	0.992
A17	25	$\sigma = 0.056 + 9.7 \times 10^{-5}\dot{\gamma}^{1.856}$	0.991
	35	$\sigma = 0.068 + 9.8 \times 10^{-5}\dot{\gamma}^{1.879}$	0.992
PP25	25	$\sigma = 0.071 + 10.5 \times 10^{-5}\dot{\gamma}^{1.860}$	0.992
	35	$\sigma = 0.067 + 10.6 \times 10^{-5}\dot{\gamma}^{1.870}$	0.992
AP25	25	$\sigma = 0.073 + 10.2 \times 10^{-5}\dot{\gamma}^{1.866}$	0.991
	35	$\sigma = 0.065 + 10.2 \times 10^{-5}\dot{\gamma}^{1.874}$	0.991
PP17	25	$\sigma = 0.059 + 9.5 \times 10^{-5}\dot{\gamma}^{1.861}$	0.992
	35	$\sigma = 0.059 + 9.6 \times 10^{-5}\dot{\gamma}^{1.861}$	0.992
AP17	25	$\sigma = 0.063 + 9.8 \times 10^{-5}\dot{\gamma}^{1.855}$	0.989
	35	$\sigma = 0.060 + 9.8 \times 10^{-5}\dot{\gamma}^{1.857}$	0.993

Kaleida Graph software was used for data analysis, T: temperature, σ_0 yield stress, K consistency index, n flow behavior index, R^2 Herschel–Bulkley model adjustment.

Table 5. Taro SFP HB model at the two levels of starch concentration and 25 °C.

SFP-Taro samples	Experimental Model	R^2
(a) SFP at 25 °C and 4.5 g	$\sigma = 0.072 + 7.520 \times 10^{-5}\dot{\gamma}^{1.865}$	0.9914
(b) SFP at 25 °C and 5.5 g	$\sigma = 0.077 + 9.31 \times 10^{-5}\dot{\gamma}^{1.840}$	0.9916

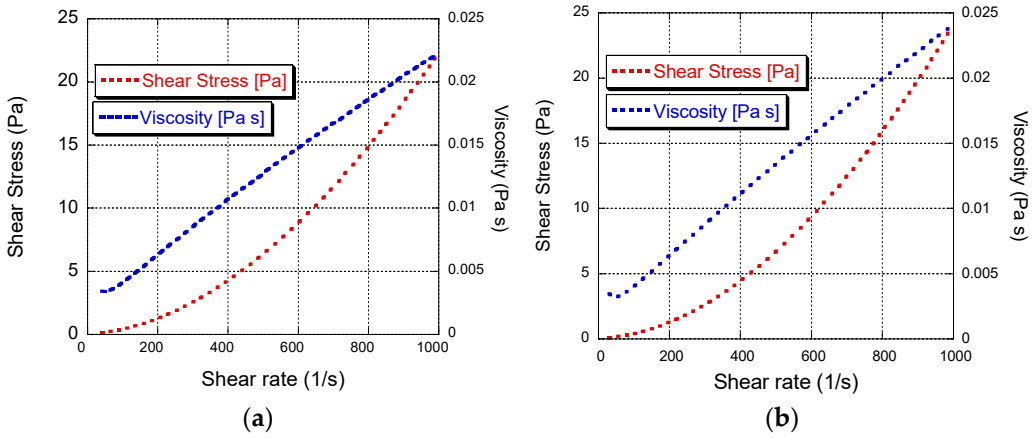

Figure 6. Rheogram of cassava SFP at 25 °C at the two levels of starch concentration (**a**) 4.5 g and (**b**) 5.5 g.

Table 6. Cassava SFP HB model at the two levels of starch concentration and 25 °C.

SFP-Cassava Samples	Experimental Model	R^2
(a) SFP at 25 °C and 4.5 g	$\sigma = 0.073 + 7.75 \times 10^{-5}\dot{\gamma}^{1.819}$	0.9873
(b) SFP at 25 °C and 5.5 g	$\sigma = 0.068 + 6.48 \times 10^{-5}\dot{\gamma}^{1.856}$	0.9922

The change that exists from the viscous (liquid) part to the elastic (solid) part was the change that exists from suspension to edible film, and the rheological models were established with which the parameters obtained can be used for transportation of large quantities of film-forming suspensions and the mass production of these edible films using

the molding method [11]. This will open a field of study for future research work that relates the physical change in more detail. Dynamic study is recommended, that is, an investigation of the storage modules and the loss module, to evaluate this change in a dynamic state.

Rheological properties of film forming solutions were not significantly affected by the starch concentration since at both levels they showed similar rheological behaviors. However, it was described in a study [42] that concentration of starch affects the rheology of the film forming suspensions, consequently affecting the resulting film properties such as tensile strength, elongation at break, water vapor permeability, and thermal properties.

The relationship between the results of the rheology is focused on the physical change that occurs in the suspension as the shear rate increases and the viscosity also increases, and a change from liquid to solid (dilatant behavior) occurs. However, with the data of Reynolds, Re numbers can be calculated from the apparent viscosity, which would be used in applications for the transport of viscous fluids and the mass production of edible films.

3.3. The Visual Aspect, the Evaluation of the Quality and the Thickness of the Films

The films of water kefir based on PP25 and AP25 without any type of treatment, only extracted and separated from the culture medium, were homogeneous, without cracks and had a color as seen in Figure 7a. For the biofilm of muscovado sugar-pineapple peel at 25 °Brix and 35 °C (PP25 at 35 °C) a mustard yellow color was observed and for the biofilm refined sugar-pineapple peel at 25 °Brix and 35 °C (AP25 at 35 °C) a beige color was observed in Figure 7b. The water kefir films that were ultrasonically and thermally treated are uniform, do not crack and have high transparency, as shown in Figure 7e,f. No differences in appearance were found for the remaining formulations. This is not a trivial aspect since the transparency, color of the films and coatings can affect the evaluation of the final product. The biofilms that were made with pure kefiran did not show an outstanding transparency [43]. Otherwise, the addition of plasticizer markedly increased the ductility of the film. Small glycerol molecules intercalate between polymer chains, breaking interpolymer bonds and separating chains, increasing membrane flexibility [40]. The edible films are flexible, easy to peel and handle. The films plasticized with 30% by weight of glycerol showed a high elasticity. Tables 7 and 8 show all biofilms studied which were approximately 1 mm thickness.

Table 8 shows the thickness of all the biofilms where it can be noticed that there is no significant difference in the samples according to Tukey–Kramer's statistical test.

The taro and cassava starch films that are illustrated in Figure 7 were homogeneous, without cracks, and presented transparency for all the starch samples. Figure 7c shows the taro edible film and Figure 7d shows the cassava edible film. In the taro edible film, a translucent light pink color can be observed due to the pigmentation of the taro starch itself.

Table 7. Thickness of water kefir grains biofilms.

Water Kefir Biofilms	Average Thickness (mm)
P25	$0.9990^{\pm 0.0016a}$
A25	$0.9981^{\pm 0.0011a}$
P17	$0.9985^{\pm 0.0016a}$
A17	$0.9974^{\pm 0.0012a}$
PP25	$0.9985^{\pm 0.0011a}$
AP25	$0.9983^{\pm 0.0014a}$
PP17	$0.9984^{\pm 0.0015a}$
AP17	$0.9983^{\pm 0.0012a}$

$^{\pm}$ Standard deviation, ^{a} Tukey–Kramer test same group.

Table 8. Thickness of edible films of starch.

Edible Films of TPS	Average Thickness (mm)
Taro 5.5 g	0.9982 $^{\pm 0.0003a}$
Taro 4.5 g	0.9984 $^{\pm 0.0006a}$
Cassava 5.5 g	0.9975 $^{\pm 0.0007a}$
Cassava 4.5 g	0.9981 $^{\pm 0.0007a}$

$^{\pm}$ Standard deviation, ^{a} Tukey–Kramer test same group.

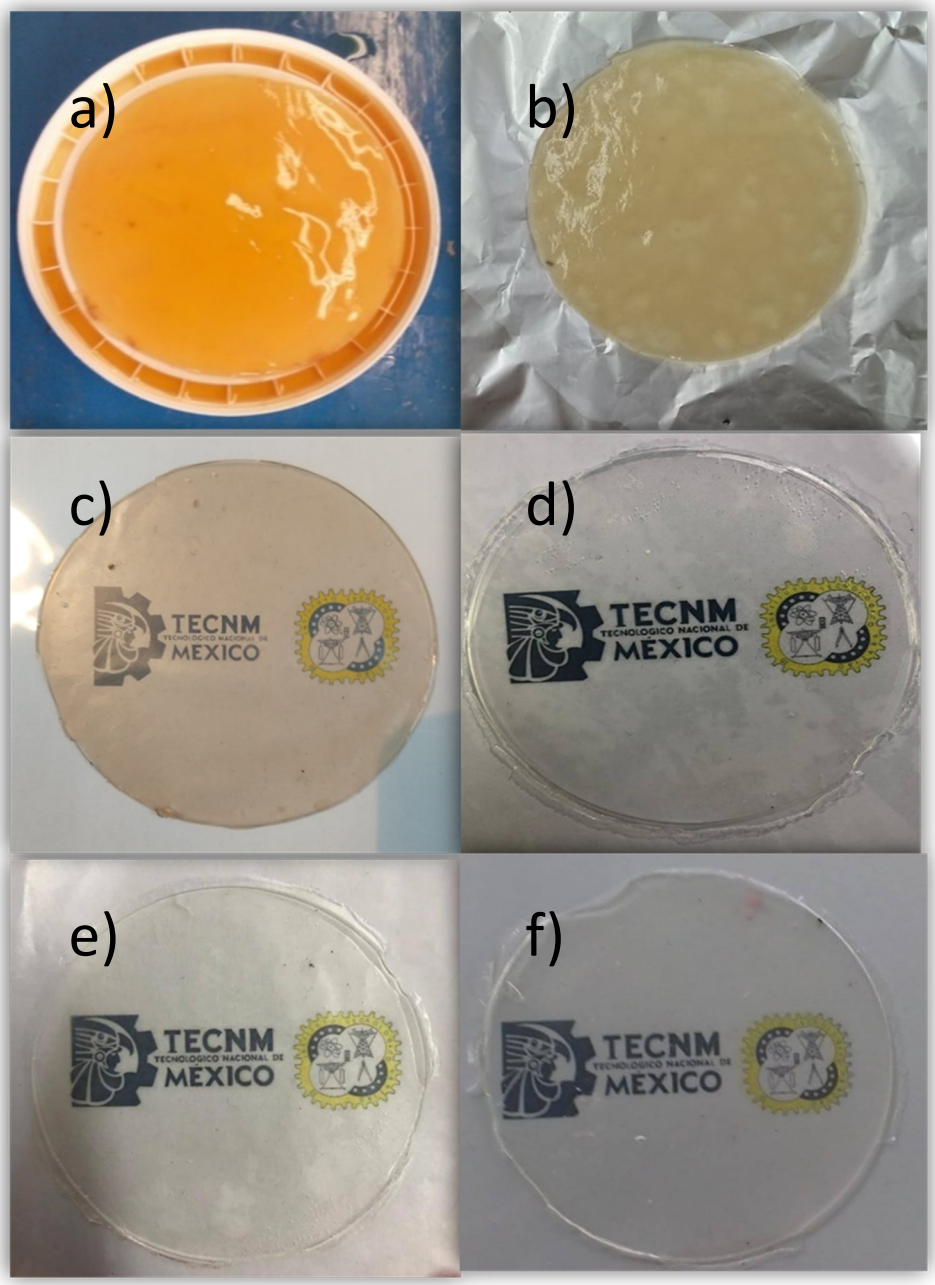

Figure 7. (**a**) Biofilm of kefir PP25 to 35 °C, (**b**) biofilm AP25 to 35 °C, (**c**) edible film of taro, (**d**) edible film of cassava, (**e**,**f**) biofilm of water kefir grains. All edible films were dry.

Once the edible films have been obtained, it is worth mentioning that reference is made to edible films in previous studies reported by different authors that indicate that many microbial polysaccharides (MPs) are better alternatives for food packaging than the polysaccharides obtained from plant and animal sources [44]. The incorporation of

bioactive components during their production has further enhanced the intrinsic features of packaging materials such it is the case for grain biofilms from water kefir grains as well as taro and cassava starch edible films [45].

3.4. Film Microstructure by Scanning Electron Microscopy (SEM)

With the intention of studying the microstructure of biofilms, microscopic images of the faces of kefir, taro and cassava, edible films were observed by scanning electron microscopy (SEM). Figure 8m,n,o shows a microscopic image of a 30% by weight glycerol plasticized kefir biofilms, in which a continuous and uniform laminated biofilm can be observed without agglomerations, voids, defects, or perforations in the membrane. The surface has an irregular shape with a diameter of 10 to 15 μm and structurally it could open and spread as a thick biofilm. Some authors have reported the existence of microbial consortia of bacteria and yeasts [46] throughout the outer layer of the biofilm, which is more densely populated by microorganisms than the inner part. At higher magnification, the outer shell of the kernel was found to contain lactobacilli, lactococci, and yeasts. The inner layer of the biofilm was similar, except that the lactobacilli were much larger, and more yeast cells were found in the inner layer of the biofilm. In all the SEMs for the biofilm tests, a smoother surface was observed; however, irregularities due to organic matter were also observed. The fractured surfaces of all cassava and taro edible films were studied by SEM (Figure 8) to assess the influence of the addition of different concentrations of starch nanocrystals on their microstructure. All edible films showed smooth fracture. No apparent phase separations were observed, confirming the homogeneous dispersion of glycerol presented in the starch matrix. As expected, the fractured surface of cassava and taro at 4.5 g starch-based biocomposite (Figure 8a–c for cassava and Figure 8g–i for taro) appeared to be more brittle with agglomeration of starch.

3.5. A_w of Kefir, Taro and Cassava Edible Films

Since water is the most ubiquitous plasticizer of hydrophilic polymers, it is important to analyze its state and the quantities present in the polymeric matrix in order to evaluate its effects on the properties of the biopolymer [31] and its microbiological action to obtain the edible values lower than 0.6 that are required for avoiding microbial development.

In Figures 9–11, the water activities of the biopolymers of kefir, taro, and cassava are shown, respectively.

The determination of the water content, whatever the precision of the analytical method, is not sufficiently informative in relation to the stability of the investigated food product. The a_w provides additional information since it accounts for the availability of water for degradation reactions [47].

The chemical and microbiological stability of a material has been qualitatively represented by means of a stability map indicating that values below 0.6 show that the microbiological stability and the development of fungal bacteria and yeasts is not allowed [48,49]. Thus, it can be seen that the deterioration reactions increase when the a_w of the product rises due to a greater mobility of the reactive water molecules, favoring their interaction to react. Figure 9 shows the water activity at different relative humidities and it is observed that at relative humidities below 57% there will be no microbial activity with a water activity value of less than 6, however at a relative humidity of 11% and at 25 °C there is still probiotic activity in the kefir biofilm.

The results demonstrated that there is microbiological stability with some atmospheres modified by salts due to their water activity since the level is below 0.6. However, unlike conventional starch films, exceeding the value of 0.6 for the biofilm of kefir grains is adequate due to its composition of probiotic microorganisms.

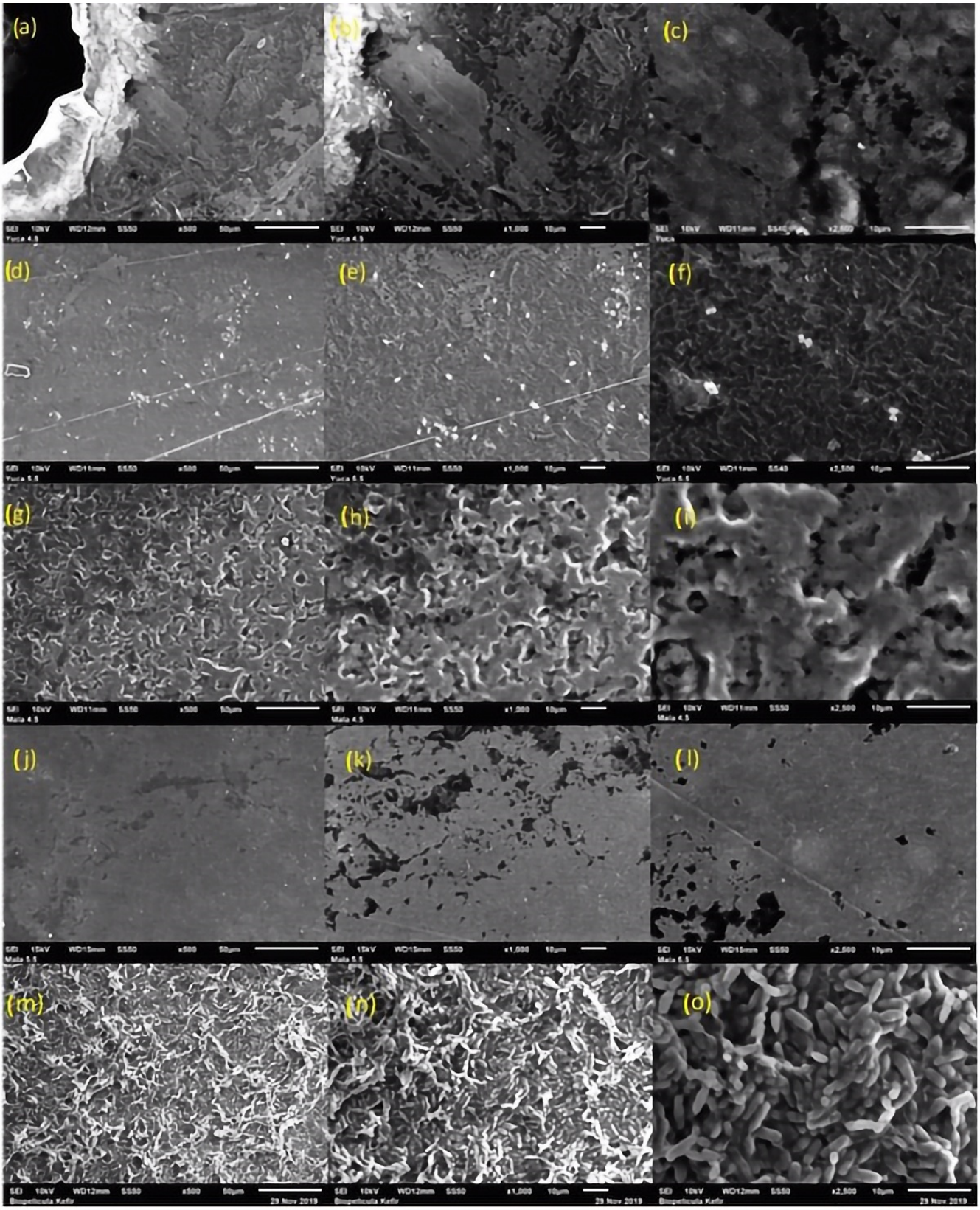

Figure 8. SEM micrograph (500, 1000, 2500×) respectively of edible films cryofracture: (**a**–**c**) cassava at 4.5 g of starch, (**d**–**f**) cassava at 5.5 g of starch, (**g**–**i**) taro at 4.5 g of starch, (**j**–**l**) taro at 5.5 g of starch, (**m**) biofilm of water kefir grains to 500×, (**n**) biofilm of water kefir grains to 1000×, (**o**) biofilm of water kefir grains to 2500×.

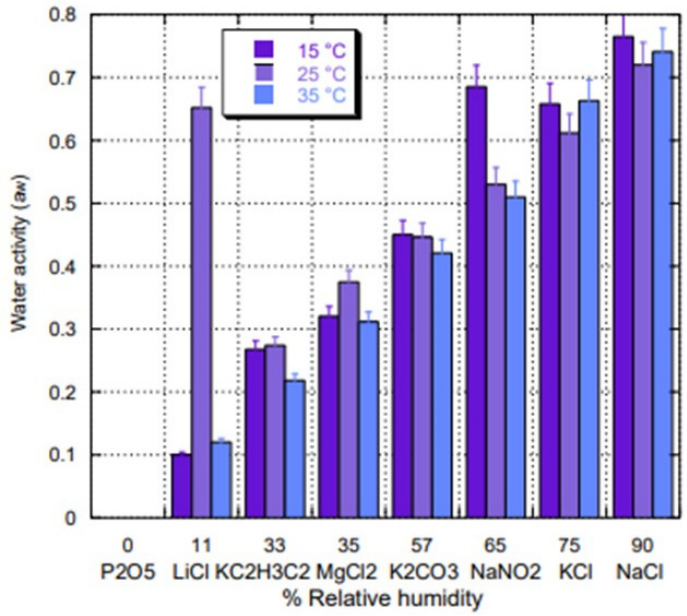

Figure 9. a_w of biofilm of kefir.

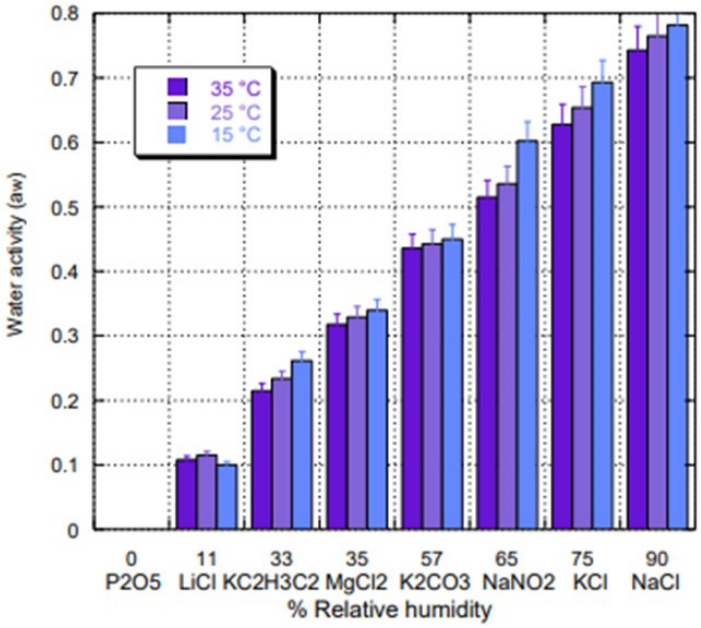

Figure 10. a_w of edible film of taro starch.

All experiments show that, if biofilms are exposed to relative humidity above 75%, there are many possibilities for microorganisms to develop in them. In Figure 9, a higher water activity is observed at low temperatures since the microorganisms present in the biofilms of kefir grains are benefited by their development at this temperature and, at higher temperatures, there is also some increased water activity. The relationship between relative humidity and temperature has not been studied more deeply; thus, a research gap is opened for future work with kefir grain biofilms. It is also observed that a relative humidity of 11% at 25 °C reached a water activity greater than 6 that will allow the development of microorganisms since these are the environmental conditions in which the water kefir grains develop better and the symbiosis between microorganisms is benefited. Due to the possibility that microorganisms could develop with these conditions of relative humidity and water activity, it is recommended in future research to evaluate the amount of water

that biofilms can adsorb and to study the culture of microorganisms, since they could grow not only probiotics but also some pathogens or fungi [49–51].

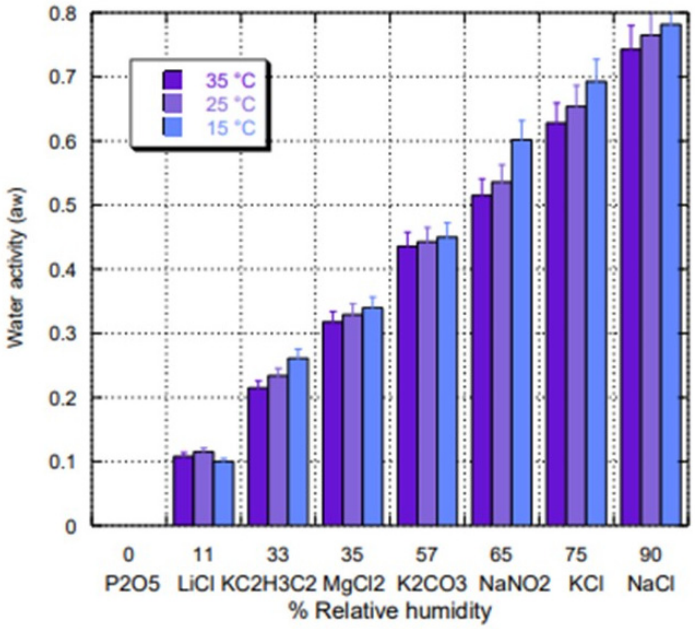

Figure 11. a_w of edible film of cassava starch.

Unlike the kefir biofilms, the TPS edible films have a normal behavior owing to the fact that there are no microorganisms present in them that form the polymeric matrix and, as seen in Figures 10 and 11, it is observed that water activity increases at higher temperatures and higher relative humidity.

During the COVID-19 pandemic, it was suggested that eating foods containing natural probiotics is beneficial for improving gut health, and thus overall health. Water kefir grains contain a broad spectrum of probiotic microorganisms, which may help improve overall health and immunity. Consequently, these can help to protect against COVID-19, especially in the elderly. Water kefir can be an important source of probiotics and prebiotics for vegans and those with dairy allergies. Water kefir grains are important for their potential health benefits [52].

Studies show that the physical properties of edible films can be affected by an antimicrobial agent, or by the addition of inorganic chemical reagents, potentially compromising the performance of the edible film and not by the use of glycerol as a plasticizer, since it can be used without damage to health. In addition, once the edible films are in a solid state, there is no migration of the agents used towards the food [53].

In this experiment, 30% wt/wt of glycerol was used as a plasticizer in relation to the starch concentration, that is, if we used two concentrations of 4.5 and 5.5 g of starch, then 1.35 g and 1.65 g of glycerol were used, respectively, for a suspension of 100 mL. The weight ratio between the starch and the plasticizer turns out to be very small, which is why, once the edible film is obtained, it does not affect the physical and chemical properties of the edible film or of the food.

3.6. Mechanical Properties of Kefir, Taro and Cassava Edible Films

The tensile strength values have already been reported by different authors [44,45,50] for starch films. For this reason, it was decided to demonstrate in this research the hardness of edible films and compare them with each other. The edible films of kefir grain, taro, and cassava starch hardness values are shown in Table 9.

Table 9. Hardness of edible films obtained.

Edible Films	Average Hardness
Kefir 3% wt/wt	57.94 $^{\pm 3.94b}$
Taro 5.5 g	90.77 $^{\pm 0.84a}$
Taro 4.5 g	91.71 $^{\pm 0.70a}$
Cassava 5.5 g	91.48 $^{\pm 1.09a}$
Cassava 4.5 g	89.82 $^{\pm 0.96a}$

$^{\pm}$ Standard deviation, ^{a} Tukey–Kramer test same group, ^{b} Tukey–Kramer test different group.

As can be seen, the biofilm of kefir grains has less hardness than conventional starch films, which indicates the possibility of more flexibility and malleability, while conventional starch films are more brittle. These flexibility and malleability tests should be measured in future experiments to verify this possibility. The results showed that the hardness rises as starch concentration increases and in the case of kefir biofilm, the hardness increases with dry matter concentration.

To elaborate on these universal scales: Shore A Hardness scale is used for measuring the hardness of flexible mold rubbers. This scale can range in hardness from very soft and flexible, medium, and somewhat flexible to hard with almost no flexibility at all. It follows that Shore A0 evidently denotes extremely soft and gel-like rubbers. While semi-rigid plastics are measured at the highest end of the Shore A Scale, Shore D Hardness Scale is reserved for measuring the hardness of hard rubbers, semi-rigid plastics, and hard plastics (Figure 12). Shore hardness proves helpful when it is necessary to choose the materials such as the type of silicone rubber to use for making a mold [54].

Once it is known how the durometer is calculated, it may still be uncertain which material or biomaterial is necessary to select for the needs of creating and replacing petroleum-derived plastics, thus, this research facilitated the selection process. Standard plastic molds are most often made with a 25–30 Shore A durometer; thus, whether conventional starch films or biofilms of water kefir grains are used as a new alternative, any material is sufficient to replace straws, bags of plastic and not so rigid materials. Figure 12 illustrates a scale of the hardness of some materials using Shore A and Shore D durometers.

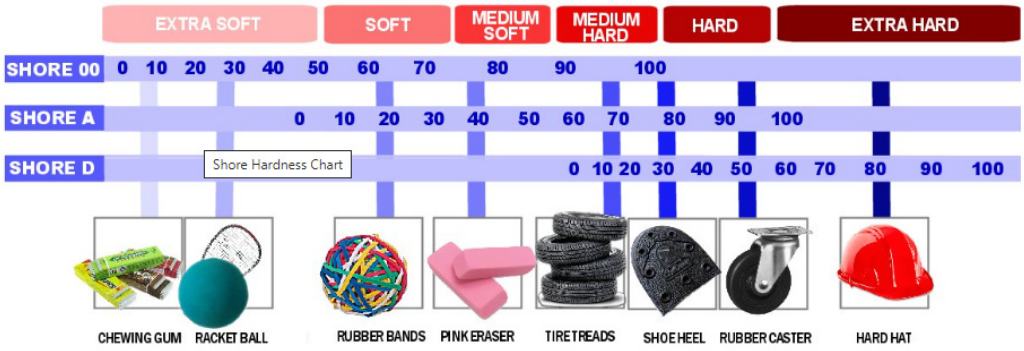

Figure 12. Shore hardness chart (sources: Smooth-on., 2019).

4. Conclusions

A new method for creating biofilms from microorganisms has been developed and compared with conventional methods such as starch films. The films obtained from whole biomass of aqueous kefir grains showed continuity, uniformity, and high transparency without cracks. As the applicability of these membranes based on aqueous kefir granules depended on their properties, it is concluded that, within the exhaustive characterization that was carried out, important parameters such as microbiological and mechanical stability properties, water activity less than 6 and similar hardness in kefir biofilms and conventional starch films, respectively, were obtained. The study of the effect of starch and dry matter

concentration of kefir grains significantly increased film stiffness. The study of the effect of starch and dry matter concentration of kefir grains significantly increased the rigidity of the film. Therefore, the optimum amount of starch concentration and raw material concentration of kefir granules as dry matter is when they are above 10 wt% in SFP. Conventional ways to develop new biodegradable materials were used to purify the film-forming agents of interest from their original biomass and perform physical or chemical modifications to enhance edible film formation. The results obtained in this study demonstrated the potential of water kefir grains to be used in the development of new biodegradable materials. The present material developed should be considered as an alternative to common sources of edible films, without the need for further purification, and these approaches could be the basis for improving its proprieties for evaluation in future investigations.

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Conflicts of Interest: The authors declare no conflict of interest.

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