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Egg White Protein Film Production Through Extrusion and Calendering Processes and its Suitability for Food Packaging Applications

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Abstract
The goal of this study was to produce a film made of egg white protein (EWP) through extrusion and calendering processes, the most common filmmaking processing technology, and to determine its potential for food packaging applications. The latter was assessed by measuring the mechanical, barrier, thermal, and optical properties; plasticizer leakage; and microbial resistance of the EWP film when exposed to specific combinations of relative humidity (RH) and temperature, and by comparing some of the results to those of commercial polylactic acid (PLA) film, the most commonly used bioplastic for food packaging applications. A transparent, continuous, thin, and uniform EWP film was produced with extruder-zone temperatures of 40 °C–50 °C–60 °C–70 °C–75 °C from feeder to die and with roller temperatures and speed set to 115–120 °C and 0.111 rpm. The permeability, lightness, and transmittance of the resulting film were affected by temperature while the RH affected its thickness, tensile properties, permeability, color, transmittance, and glycerol loss. Compared to the PLA film, the EWP film was less breakable and flexible, and had a lower barrier to water and higher rigidity, thermal resistance, and barrier to oxygen. The two materials present similar transparency, lightness, color, barrier to ethanol, and sensitivity to RH. This study proves that EWP film can be produced through extrusion and calendaring processes and can be used as an alternative to other materials for food packaging applications where thermal resistance, rigidity, strength, barrier to oxygen, and avoidance of condensation are desired.

Keywords Egg white protein · Film development · Extrusion · Calendering · Temperature and relative humidity · Food packaging applications

Introduction

Due to the economic and environmental issues surrounding petroleum-based plastics, there has been an increased pressure to find more sustainable plastics. This move towards sustainability has stimulated research in the field of bioplastics. Plant and animal proteins are readily available and have been proven to be adequate raw materials for bioplastic development (Hernandez-Izquierdo and Krochta 2008). Recent studies have shown the potential of egg white protein (EWP) as raw material for the development of a highly transparent bioplastic (Jerez et al. 2007; Lee et al. 2013; Martínez et al. 2013; Félix et al. 2014) and of composites (Peng et al. 2017). These studies have focused on the development of EWP bioplastic by compression molding and injection molding processes and of EWP composites by casting.

Despite the extrusion process being the most common processing method for plastics (Selke and Culter 2016), limited information is available on the extrusion of EWP. There is only one study related to EWP extrusion that investigated the use of extrusion to produce EWP-starch bioplastic (González-Gutiérrez et al. 2011). Therefore, to the best of our knowledge, there is no information in the literature about obtaining neat EWP bioplastic using extrusion. Furthermore,

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the combination of extrusion with other processing methods to obtain EWP film has not yet been reported. The filmmaking process commonly comprises extrusion and then chill roll or calendaring (Selke and Culter 2016). Thus, the appropriate extrusion and calendering conditions for the development of EWP films for industry adaptation are yet to be determined. Knowing these conditions would not only allow the production of EWP films by one of the processing technologies most commonly used by the packaging industry to produce films but would help to build the necessary background for production of bioplastic films made of other naturally occurring resources.

Numerous studies have been carried out to develop protein-based bioplastics due to the increased attention that bioplastics are receiving as materials for food packaging applications ranging from plain materials that can be either edible or not to the latest materials that carry active compounds (Li and Ye 2017; Li et al. 2018). In general, most of the published literature regarding protein-based bioplastics, specifically EWP bioplastics, focuses primarily on new processing approaches for their development (Félix et al. 2014; Fernández-Espada et al. 2013; González-Gutiérrez et al. 2011; Jerez et al. 2007; Jones et al. 2013; Lee et al. 2013; López-Castejón et al. 2015; López-Castejón et al. 2016; Martínez et al. 2013; Zink et al. 2016; Coltelli et al. 2016). The use of this new generation of plastics for such applications requires the understanding of the interactions occurring between food and materials as well as the effect of the surrounding environment (i.e., humidity, temperature). This understanding is needed since these interactions result in changes in the packaging material (e.g., permeability, thermal properties, mechanical properties) that result in changes in the quality and safety of the food product (e.g., flavor, texture, weight loss, microbial growth). However, the vast majority of developed bioplastics are characterized after exposure to only one specific combination of temperature and relative humidity (approx. 23 °C and 55% RH) (Fabra et al. 2015; Moro et al. 2017; Toro-Márquez et al. 2018; Valencia-Sullca et al. 2018). Therefore, any information on changes in protein-based films caused by humidity and/or temperature can help the understanding of current opportunities and constraints of this new generation of plastics as materials for food packaging applications.

Recognizing the lack of knowledge in the two above-mentioned areas, the goals of this study were as follows: (1) to identify the conditions for EWP filmmaking through extrusion and calendering processes, and (2) to investigate the current possibilities and limitations of the produced film as a food packaging material by inquiring into the effects of both temperature (4 °C and 23 °C) and relative humidity (0%, 55%, and 95%) on its thermal, mechanical, barrier, and optical properties as well as its fungal resistance and plasticizer leakage; and by comparing the film with the most commonly used bioplastic in food packaging, poly(lactic acid) (PLA).

Materials and Methods

Materials

Spray dried egg white protein (EWP) powder (82.1 ± 0.61% protein, 0.37 ± 0.17% fat, 6.04 ± 0.20% ash, and 3.51 ± 0.22% moisture determined by proximate analysis) was obtained from Rose Acre Inc. (Seymour, IN, USA). Food grade vegetable glycerin (GLY) was obtained from Starwest Botanicals (Sacramento, CA, USA). Magnesium nitrate ($Mg(NO_3)_2$) and potassium sulfate (K_2SO_4) were purchased from Columbus Chemical Industries Inc. (Columbus, WI, USA). Desiccant was obtained from W.A. Hammond Drierite Co. Ltd. (Xenia, OH, USA) and ethanol (purity ≥ 99.5%) from VWR International (Radnor, PA, USA). Potato dextrose agar, free glycerol reagent, and glycerol standard solution, all food grades, were purchased from Sigma-Aldrich Co. LLC. (Saint Louis, MO, USA). Single spore isolates of *Aspergillus niger* and *Penicillium pinophilum* were isolated from spoiled food. Polylactic acid (PLA) film (44-μm thickness) was obtained from EVLON EV-HS1 (BI-AX International Inc., Wingham, ON, Canada).

Preparation of EWP Films

EWP films were produced by combining extrusion and calendering processes. Extrusion was used to obtain EWP extrudates while calendaring was used to flatten the extrudates into films to mimic current plastic processing practices. EWP powder and a mixture of water and glycerol in 2:1:1 ratio were introduced in a co-rotating twin-screw extruder (Model MP19TC2-25, APV Baker; Grand Rapids, MI, USA) with a screw speed of 150 rpm using a gravimetric feeder (Model K2V-T20, K-Tron; Pitman, NJ, USA) and a pump (Model E2 Metripump, Brook Crompton; Hudders Field, England), respectively. The different zones of the extruder were set to various temperature profiles until the desired extrudates were obtained. The resulting extrudates were immediately placed between two Teflon sheets (McMaster-Carr, Elmhurst, IL, USA) and then calendered using double drum dryers (Overton Machine Company, Dowagiac, Michigan, USA). Different temperatures and speeds for the drum dryers were investigated. The resulting films were peeled off from the Teflon sheets after cooling down for 2 min at room temperature. Mixing of materials and processing were performed three times to obtain three batches of EWP films (replicates). Figure 1 illustrates the preparation of the EWP films.

Storage Conditions

EWP films were conditioned in six different environments for 48 h prior to characterization. The different environments (conditioning treatments) resulted from the combination of

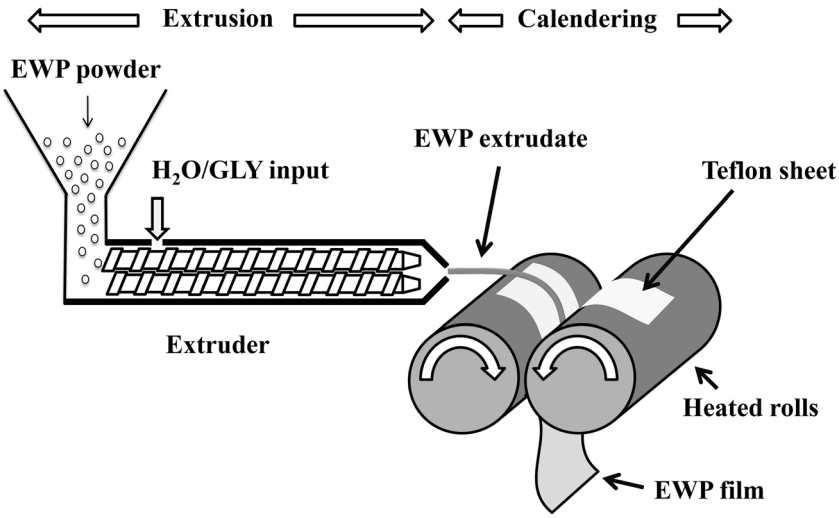

Fig. 1 Preparation of EWP films by extrusion and calendering processes

two different temperatures (4 °C or 23 °C) and three different RH (0%, 55%, or 95%). A constant temperature and humidity room set at 23 °C and a walk-in test chamber set at 4 °C were used to obtain the two temperatures. K_2SO_4 and $Mg(NO_3)_2$ saturated solutions and desiccant were placed inside buckets with air-tight lids to create environments of 95%, 55%, and 0% RH at both temperatures, except for 55% RH at 23 °C since this was the RH at which the above-mentioned room was set.

EWP Film Characterization

Thickness Determination

The thickness of the EWP films after exposure to the different conditioning treatments was obtained by averaging the measurements from five random film positions obtained using an electronic digital micrometer (Fowler® 0-1" Digital Counter Micrometer, Port Washington, NY, USA). The results were expressed in μm.

Thermal Characterization

The second-order transition temperature of each EWP film after exposure to the different conditioning treatments was determined using a differential scanning calorimeter (DSC Q100; TA Instruments, Newcastle, DE, USA) with a liquid nitrogen cooling system. An amount between 6 and 10 mg of each EWP film was hermetically sealed in an aluminum pan (TA Instruments, Newcastle, DE, USA), cooled to 0 °C, and then heated to 300 °C at a rate of 30 °C min^{-1} under N_2 atmosphere. TA analysis software was used to do the data analysis in accordance with the ASTM Method D3418-12 (ASTM 2012a). Two samples from each conditioning treatment, each from a different replicate, were analyzed.

Mechanical Characterization

EWP films were cut into rectangular strips of 0.1 × 0.01 m and then exposed to the different conditioning treatments. PLA films were cut into rectangular strips of the same dimensions and exposed to 55% RH and 23 °C. The elongation at break (E_b), tensile strength (σ_{max}), and modulus of elasticity (E) of each strip were measured according to the ASTM method D882-12 (ASTM 2012b) using an Instron Universal Testing Machine UTS SFM–20 (United Calibration Corporation, Huntington Beach, CA, USA) with a load cell of 5 kN. A speed of 0.01 m s^{-1} and an initial grip separation of 0.05 m were used. At least six strips from each of the three replicates of each conditioning treatment were evaluated.

Barrier Characterization

Water Vapor Permeability The water vapor transmission rate (WVTR) of the EWP films after exposure to the different conditioning treatments was measured using a Mocon PERMATRAN® W Model 3/33 Water Permeability Analyzer (Minneapolis, MN, USA) according to the ASTM Method F1249-13 (ASTM 2013a). Film samples were double-masked with sticky aluminum foil, leaving a circular uncovered film area of 0.4×10^{-4} m^2. Testing for samples stored at 4 °C was performed at 10 °C which is the lowest temperature the equipment can reach. The samples stored at 23 °C were analyzed at that same temperature. The RH of one side of the cell was matched to that of the storage conditions (55% or 95%) while the RH of the other side of the cell was 0%. The WVP (kg m m^{-2} s^{-1} Pa^{-1}) of the EWP films was calculated using the following equation:

$$\mathrm{WVP} = \frac{\mathrm{WVTR} \times L}{\Delta P}$$

where L is the mean film thickness (m) and ΔP is the partial water vapor pressure difference (Pa). The permeability of three samples from each conditioning treatment, each from a different replicate, was measured.

Oxygen Permeability The oxygen transmission rate (OTR) of the EWP films after exposure to the different conditioning treatments was measured using a Mocon OX-TRAN® Model 2/21 (Minneapolis, MN, USA) according to the ASTM method D3985-05 (ASTM 2005). Film samples were double-masked with sticky aluminum foil, leaving a circular uncovered film area of 0.4×10^{-4} m^2. Testing for samples stored at 4 °C was performed at 10 °C which is the lowest temperature the equipment can reach. The samples stored at 23 °C were analyzed at that same temperature. RH was matched with that of the storage conditions (0%, 55%, or 95%). Samples stored at 95% RH were tested for permeability at 90% RH because this is the highest RH the equipment sensor can perform. The oxygen permeability (OP) (kg m m^{-2} s^{-1} Pa^{-1}) of the EWP films was calculated using the following equation:

$$\mathrm{OP} = \frac{\mathrm{OTR} \times L}{\Delta P}$$

where L is the mean film thickness (m) and ΔP is the partial oxygen pressure difference (Pa). The permeability of three samples from each conditioning treatment, each from a different replicate, was measured.

Ethanol Permeability The ethanol permeation rate of the EWP films was measured using permeation cells made from poly(methylmethacrylate). The film samples were double-masked with sticky aluminum foil, leaving a circular uncovered film area of 0.3×10^{-3} m^2. Cells were filled with 1 mL of ethanol and then placed inside buckets and moved to the chambers at 4 °C or 23 °C. The buckets were modified with inlet-outlet ports that allowed the air from a cylinder to pass through them to remove from their headspace the ethanol permeated from the cells during storage. Precision needle valves were used to maintain a constant airflow rate of about 200 mL min^{-1}. The RH of the bucket headspace was controlled by humidifying the air of the inlet-port using gas washing bottles containing K_2SO_4 and $Mg(NO_3)_2$ saturated solutions. The experiments at 0% RH were performed in modified buckets containing desiccant. The experiment at 55% RH and 23 °C was performed in a conditioning room. The weights of the cells were recorded daily using an analytical balance (OHAUS Corp., Parsippany, NJ, USA). Linear regression-derived slopes of the steady state (linear) portion of weight loss versus time curves were used to estimate the ethanol transmission rate (EtOHTR). The ethanol permeability (EtOHP) (kg m m^{-2} s^{-1} Pa^{-1}) of the EWP films was calculated using the following equation:

$$\mathrm{EtOHP} = \frac{\mathrm{EtOHTR} \times L}{\Delta P}$$

where L is the mean film thickness (m) and ΔP is the partial ethanol pressure difference (Pa). The permeability of three samples from each conditioning treatment, each from a different replicate, was measured.

Optical Characterization

Color The color of the EWP films after exposure to the different conditioning treatments and of the PLA film after exposure to 55% RH and 23 °C was measured using a Labscan XE colorimeter (Hunter Laboratories, Reston, VA, USA) and characterized using the CIE $L^*a^*b^*$ system. The variable L^* represents lightness ranging from 0 to 100%. The variable a^* ranges from green (negative) to red (positive) and variable b^* ranges from blue (negative) to yellow (positive). Five measurements from each replicate from each conditioning treatment were taken.

Transmittance The transmittance (%) of the EWP films after exposure to the different conditioning treatments and of the PLA film after exposure to 55% RH and 23 °C were measured using a spectrophotometer (Lambda 25 UV/VIS Spectrometer; PerkinElmer Instruments, Waltham, MA, USA) equipped with an integrating sphere in the spectral range from 300 to 850 nm and with a scan speed of 480 nm min^{-1}. The transmittance values obtained at a wavelength of 600 nm were reported. Five measurements from each replicate from each conditioning treatment were taken.

Fungal Resistance

The fungal resistance of the EWP films after exposure to the different conditioning treatments was measured according to a slightly modified ASTM Method G21-13 (ASTM 2013b). 2.5 × 2.5 cm pieces of EWP film were placed on potato dextrose agar in plastic petri dishes (9-cm diameter). One hundred microliters inoculum (1×10^6 spores/mL) of *Aspergillus niger* (ATCC 9642) and *Penicillium pinophilum* (ATCC 11797) was deposited on the surface of the EWP films, and the petri dishes were closed with their lids to avoid external contamination and to allow their manipulation when measuring fungal colony size. Culture preparation was performed as described in Almenar et al. (2007). The fungi were provided by the microbiology laboratory of the department of Food Science and Human Nutrition and the department of plant pathology at Michigan State University. Fungal spores were confirmed using a Bright-Line hemacytometer (Hausser Scientific,

Horsham, PA, USA). Petri dishes containing conidial suspensions without the films were used as controls. Controls and treatments were incubated at 23 °C and 55% RH for 48 h. Growth of the cultures in both controls and treatments was evaluated by measuring the diameter of the colony on the film and agar surface. Measurement of diameters was made using a conventional ruler. These measurements could be made without problem due to the optical transparency of both the agar and Petri dish. One sample from each replicate (three samples total) from each conditioning treatment was evaluated.

Plasticizer Leakage

Glycerol leakage from the EWP films into water was quantified to determine the effect of the different storage conditions on plasticizer loss from the polymer matrix. Films after exposure to the different conditioning treatments were cut into 1 × 1 cm pieces and were then weighed. Each piece was immersed into 50 mL of distilled water for 24 h at 23 °C. After removal of the piece, the resulting solution was vigorously shaken and a sample was taken. Samples were used for quantitative enzymatic determination of glycerol. Free glycerol reagent, glycerol standard solution, and a UV-Vis spectrophotometer (Perkin-Elmer Lambda 25, Perkin Elmer Instruments, Waltham, MA, USA) were used to carry out the enzymatic assay following the protocol of the manufacturer. Glycerol leakage into water was calculated as follows:

$$\text{Gly Content (mg Gly/mg film)} = \left[\frac{(A_{\text{Sample}} - A_{\text{Blank}})}{(A_{\text{Standard}} - A_{\text{Blank}})} \times \text{CS}\right] \times 50\ \text{mL/weight of film (mg)}$$

where Gly is glycerol, A is absorbance, and CS is concentration of standard (0.26 mg Gly mL^{-1}). Three different films for each conditioning treatment were used.

Statistical Analysis

IBM SPSS 25 (IBM Corporation, Armonk, NY, USA) was used to perform a two-way analysis of variance with interaction (ANOVA; Levene's test; Tukey's test; $P \leq 0.05$) between the means to determine the effect of temperature (4 °C or 23 °C) and RH (0%, 55%, or 95% RH) on each of the investigated film properties and to determine interactions between temperature and RH. The multiple comparisons for T*RH interactions were pairwise comparisons and gave the mean differences between RH values within each temperature and between temperatures within each RH value.

Results and Discussion

Extrusion and Calendering Processing Conditions for EWP Film Development

The adequate extrusion and calendering processing conditions to obtain EWP films were determined from trial-and-error experiments. Table 1 summarizes the different combinations of processing conditions used for both extrusion and calendering during the various experimental trials. Desired EWP extrudates were obtained when the five different temperature zones of the extruder from feeder to die were 40 °C–50 °C–60 °C–70 °C–75 °C. Films with desired properties were obtained from the extrudates when the drum dryers were heated at 115–120 °C and its rotational speed was 0.111 rpm. The resulting films were transparent, continuous, and uniform, and had a thickness of 110 ± 13 μm.

Table 1 Extrusion and calendering processing conditions used to obtain EWP films

Extrusion	Calendering		Results
Temperature profile (extruder feed zone–die) (°C)	Drum dryer temperature (°C)	Rotational speed (rpm)	
50–80–80–100–100			Extrudates with hard texture that could not be calendered.
40–40–40–50–60			Liquid-like extrudates.
40–50–65–75–80			Failed to obtain uniform shaped extrudates due to overheating of extruder's die.
40–50–60–70–75	150	0.33	Cloudy EWP films.
40–50–60–70–75	150	0.167	Transparent EWP films with breakage in the center of the sheet.
40–50–60–70–75	115–120	0.111	Transparent, continuous, and uniform EWP films.

Effects of Temperature and Relative Humidity on EWP Film Properties

Thickness

The thickness of the EWP films after exposure to different conditioning treatments ranged between 97 and 123 μm (Table 2). RH had a significant effect on this variation ($P = 0.006$; Table 3) while temperature did not ($P = 0.084$; Table 3). The higher the RH, the thinner the EWP film was based on means for groups in homogeneous subsets (post hoc test). This phenomenon can be attributed to a loss of glycerol with increasing RH that led to more bonding between polymer chains reducing free volume. The loss of glycerol occurred due to a decrease in glycerol content compared to water content caused by the capability of a glycerol-water solution to gain moisture until reaching equilibrium with environmental moisture. This led to a rise in the relative vapor pressure of the glycerol-water solution on the film surface that facilitated the leach out of both water and glycerol from the film. The dependence of the relative vapor pressure of a glycerol-water solution on its glycerol content has previously been reported (Glycerine Producers' Association 1963). The decreased film thickness associated with increasing RH was not observed for films stored at 23 °C and 95% (Table 2) due to a two-way interaction between temperature and RH ($P = 0.003$; Table 3). The higher temperature led to water molecules with an increased vapor pressure (Kessler 2006). Therefore, there were more water molecules surrounding the EWP films at 95% RH during storage at 23 °C compared to 4 °C. This resulted in absorption of water molecules by the EWP polymer matrix high enough to substitute for the loss of glycerol observed in films exposed to other storage conditions. PLA, the most commonly used bioplastic in food packaging, also presents higher moisture sorption at high RH (Holm et al. 2006).

Plasticizer Leakage Into Water

The amounts of glycerol leaked from the EWP films into water after exposure to different conditioning treatments (Table 2) only varied with RH ($P = 0.031$ for RH; Table 3). The higher the RH, the lower the amount of glycerol in water based on means for groups in homogeneous subsets. This phenomenon can be attributed to the higher loss of glycerol the films experienced during storage. The higher the RH to which the EWP films were exposed, the greater the glycerol loss from the films during conditioning and consequently, the lesser the glycerol available to leak into the water upon subsequent testing. These results are in agreement with the thickness results presented in the "Thickness" section and support the claimed loss of glycerol with increasing RH. The amounts of glycerol leaked from EWP films into water constituted more than 80% of the original glycerol content of the films. These values are higher than those previously reported in the literature for other protein-based films (Wakai and Almenar 2015). According to the U.S. Food and Drug Administration Code of Federal Regulations Title 21 Section 582.1320, glycerol is a substance that is generally recognized as safe when used in accordance with good manufacturing or feeding practices. Although high glycerol migration may be acceptable depending on the intended application, the substantial glycerol loss from the EWP polymer matrix would most likely result in films with undesirable properties (e.g., mechanical). Therefore, these results suggest that the EWP film is not an adequate material to package water-based liquid food products. In contrast, the minimal loss of glycerol during conditioning, especially at 55% RH or lower, indicates that the EWP film is a good choice for semi-solid and solid food products, especially for those with a low water activity. Aging studies performed on compressed protein-based bioplastics containing glycerol as plasticizer have shown mechanical stability over long storage periods (3–4 months) that has been

Table 2 Thickness, glycerol leakage into water, and first- and second second-order transition temperatures of EWP films after exposure to different conditioning treatments

Temperature (°C)	RH (%)	Thickness (μm)	Leaked Gly (mg Gly mg^{-1} film)	First second-order transition temperature (°C)	Second second-order transition temperature (°C)
4	0	122.3 ± 25.4^{Aa}	0.331 ± 0.034^{Aa}	147.7 ± 5.4^{Aa}	261.0 ± 2.3^{Aa}
	55	105.2 ± 22.7^{Ba}	0.313 ± 0.032^{ABa}	143.4 ± 0.5^{Aa}	264.9 ± 2.6^{ABa}
	95	96.6 ± 19.2^{Ba}	0.278 ± 0.012^{Ba}	139.4 ± 2.5^{Aa}	267.7 ± 4.7^{Ba}
23	0	117.4 ± 12.1^{ABa}	0.353 ± 0.008^{Aa}	128.2 ± 17.3^{Aa}	265.9 ± 0.1^{Aa}
	55	104.3 ± 8.5^{Aa}	0.322 ± 0.022^{Aa}	148.5 ± 3.5^{Bb}	260.4 ± 0.8^{ABa}
	95	122.7 ± 12.7^{Bb}	0.318 ± 0.029^{Aa}	150.0 ± 3.5^{Bb}	259.7 ± 1.9^{Bb}

[1] Different uppercase letters and lowercase letters indicate significant differences ($P \leq 0.05$) caused by RH and temperature, respectively

Table 3 *P* values resulting from Univariate ANOVA (SPSS) (*$P \leq 0.05$ indicates the single or combined (interaction) effect of the factors temperature (*T*) and relative humidity (RH) on each variable)

Factor	Variable																	
	Fungal growth		Thickness	Tensile			Permeability			Color			Transmittance					
	P. pinophilum	*A. niger*		Break elongation (E_b)	Tensile strength (σ_{max})	Modulus of elasticity (*E*)	Water vapor	Oxygen	Ethanol	*L*	*a**	*b**		First second-order transition	Second second-order transition	Glycerol loss
T	0.377		0.031*	0.084	0.136	0.508	0.321	0.015*	0.000*	0.005*	0.049*	0.483	0.668	0.000*	0.793	0.130	0.068
RH	0.321		0.735	0.006*	0.000*	0.020*	0.006*	0.000*	0.000*	0.000*	0.424	0.000*	0.000*	0.000*	0.355	0.832	0.031*
T*RH	0.253		0.353	0.003*	0.016*	0.260	0.003*	0.004*	0.000*	0.005*	0.047*	0.687	0.455	0.003*	0.070	0.027*	0.558

attributed to a little loss of glycerol (Cunningham et al. 2000; Orliac et al. 2003). These results along with our results encourage trials of food packaged in EWP film due to the expected stability of the film properties during food storage.

Thermal Properties

Figure 2 presents the DSC thermograms of the EWP films after exposure to different conditioning treatments. They show two endothermic peaks for the EWP films that are associated with second-order transition temperatures. The first second-order transition temperature of the films occurred at approximately 150 °C (Table 2). Similarly, Lee et al. (2013) reported a first second-order transition temperature of around 150 °C for compressed EWP sheets. The second second-order transition temperature of the EWP films occurred between 260 and 270 °C (Table 2). Neither temperature nor RH had an effect on either of the second-order transition temperatures of the EWP films (Table 3). However, an interaction between temperature and RH ($P = 0.027$; Table 3) resulted in an increased second second-order transition temperature with increasing RH at 4 °C and vice versa at 23 °C. This difference can be attributed to the films differing in moisture content because of their exposure during conditioning to different amounts of water molecules as water vapor pressure increases with increasing temperature (Kessler 2006). A lower exposure to water molecules and consequently, less absorption of these, resulted in a more heat-stable film. Based on these results, EWP films obtained by extrusion and calendering processes have a thermal resistance that makes them suitable for food treatments like sterilization, processing, and cooking up to 140–150 °C. The higher thermal resistance of the EWP film compared to the PLA film (T_g of 57 °C (Joo et al. 2011)) makes the former suitable for food treatments unfit for PLA like microwave cooking.

Mechanical Properties

EWP films had an E_b ranging from 16.2 to 37.7%, a σ_{max} between 3.4 and 4.2 MPa, and an *E* between 40.7 and 85.4 MPa (Fig. 3). These values are lower for E_b and σ_{max}, and higher for *E* than those reported for compressed EWP bioplastic in the literature (González-Gutiérrez et al. 2010; Lee et al. 2013). The different values can be attributed to the lower GLY content of our films compared to the compressed sheets (25% vs. 35% to 45%). The effect of glycerol content on the tensile properties of EWP has been reported previously (Lee et al. 2013). Comparing the mechanical properties between EWP film and PLA film at the same storage conditions of 23 °C and 55% RH shows that the EWP film is less flexible (37.7% compared to 163.5%), less breakable (4.0 MPa compared to 0.11 MPa) and more rigid (43.6 MPa compared to

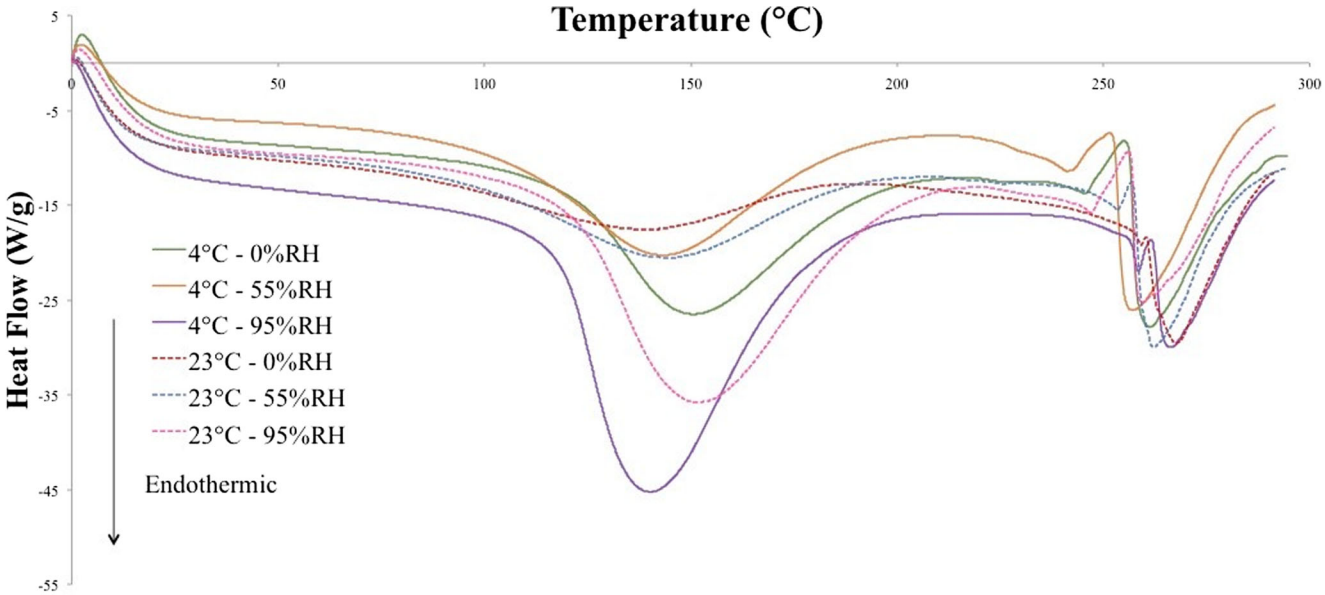

Fig. 2 DSC thermograms of EWP films after exposure to different conditioning treatments

3.96 MPa) (Fig. 3). These results suggest that EWP film is a good alternative to PLA film for food packaging applications when rigidity and strength to protect the food product are desired (e.g., produce packaging). RH had an effect on the mechanical properties of the EWP films while temperature did not (Table 3). EWP films stored at 95% RH were significantly less flexible ($P \leq 0.05$) than EWP films stored at or below 55% RH at both storage temperatures. This can be attributed to the loss of glycerol with increasing RH, as discussed previously. The decrease in E_b with decreasing glycerol content has also been reported for EWP sheets (Lee et al. 2013). The E_b of the EWP films almost halved with an increase in RH from 0 to 95% at both storage temperatures. The EWP films exposed to 0% and 55% RH did differ in flexibility when stored at 23 °C but not at 4 °C due to an interaction between temperature and RH ($P = 0.016$; Table 3). Loss of elongation has also been reported for PLA films when exposed to higher temperatures and RH (Ho et al. 1999; Holm et al. 2006).

A significant ($P = 0.006$; Table 3) change of the E of the EWP films with RH was observed at both storage temperatures as well. The EWP films were less rigid and stiff with a rise in RH, except for those stored at 4 °C and 95% RH due to an interaction between temperature and RH ($P = 0.003$; Table 3). An increase in rigidity and stiffness would have been expected due to the loss of plasticizer with increasing RH. However, the specific rearrangement of the protein network due to the new bonds caused by the loss of glycerol led to a less rigid and stiff protein matrix. Decrease in E with increasing moisture content has also been reported for PLA films exposed to RH between 58 and 98% at 25 °C. This decrease has been attributed to the plasticization of the material by water (Holm et al. 2006). Other bio-based plastics have also shown the same reduced rigidity with increasing RH (Chang et al. 2000). The σ_{max} of the EWP films also decreased as the RH increased ($P = 0.020$). The EWP film was approximately 15% more breakable with a rise in RH from 0 to 95% ($P \leq 0.05$). The lower σ_{max} was due to the less free volume between the polymer chains caused by the loss of glycerol with increasing RH. Similarly, López-Castejón et al. (2015) observed a lower σ_{max} in compressed EWP sheet when reducing the amount of glycerol. The tensile strength of the PLA film also decreases with increasing RH. This phenomenon has been attributed to rapid initial moisture absorption (Holm et al. 2006). Based on the above results, EWP films show the most appropriate properties for food packaging applications (more flexibility, less breakability, and less rigidity) when being stored at 55% RH and 23 °C.

Barrier Properties

The WVP of the EWP films ranged between 0.3×10^{-12} and 3.9×10^{-12} kg m m^{-2} s^{-1} Pa^{-1} (Table 4) due to the impact of both temperature and RH (Table 3). At 95% RH, the WVP of the EWP films was higher ($P \leq 0.05$) at 4 °C than at 23 °C but not at 55% RH due to an interaction between temperature and RH ($P = 0.004$; Table 3). This finding can be explained by the more water at 95% RH along with the negative heat of sorption (take up of more water at lower temperatures) at constant RH that characterizes most foods and biomaterials (Bell and Labuza 2000) since permeability of bio-based films increases with the increases in their water contents due to the plasticizing effect of water (Vieira et al. 2011). RH also affected the WVP of the EWP films ($P = 0.000$; Table 3). At 95% RH, the WVP of the EWP films was one order of magnitude higher ($P \leq 0.05$) than at 55% RH regardless of temperature. This

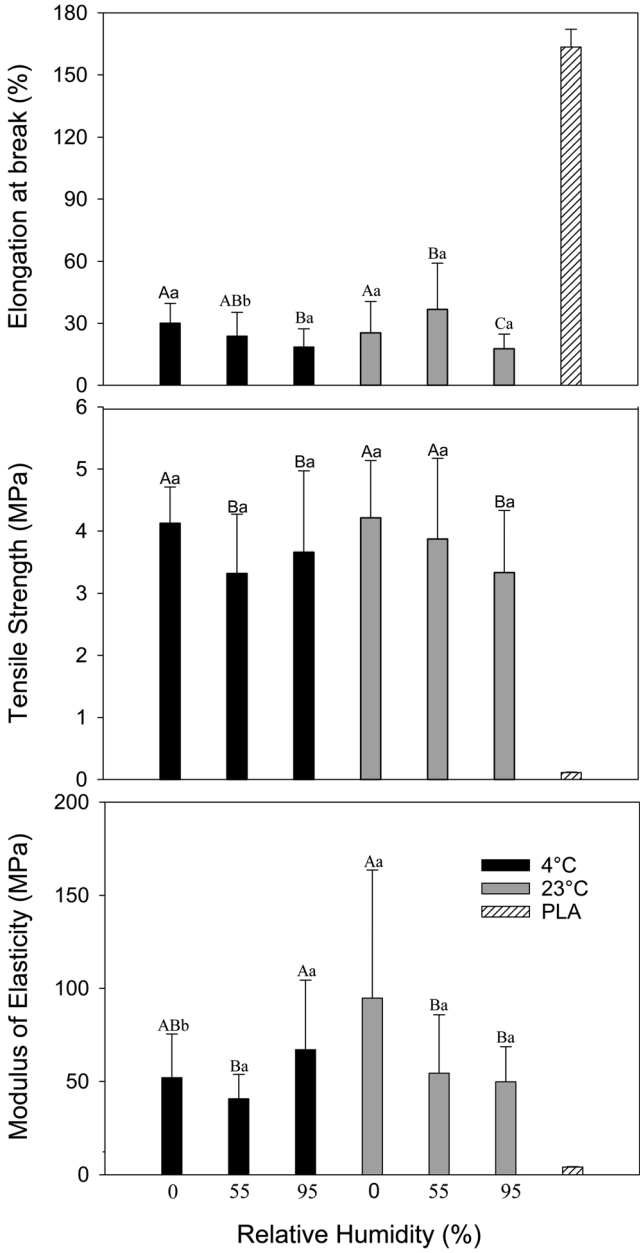

Fig. 3 Tensile properties of EWP films after exposure to different conditioning treatments

higher permeability can be attributed to the greater loss of glycerol that occurred during conditioning with increasing RH. Since glycerol attracts water from the environment and holds it (Glycerine Producers' Association 1963), a film with a lower amount of glycerol would facilitate the movement of water molecules through its matrix (higher water diffusion) resulting in an increase in water permeability. Similarly, Miller and Krochta (1997) reported higher diffusion of permeants through edible films with a decrease in glycerol content. The WVP of EWP film at 95% RH and 23 °C was 2.15×10^{-12} kg m m^{-2} s^{-1} Pa^{-1}. This value is two orders of magnitude higher than that of PLA under the same conditions (Almenar and Auras 2010; Joo et al. 2011; González-Buesa et al. 2014). Therefore, the EWP film is not a desired material to package food that requires a good barrier to water for shelf-life extension, but it is a good choice for allowing water to escape from the package to avoid condensation that causes spoilage (e.g., fungal growth) in some types of food categories like produce.

Both temperature and RH also had significant effects on the OP of the EWP films (Table 3). Significant differences in OP resulting from changes in temperature and RH were observed in the EWP films stored at higher RH (Table 4). In fact, when the OP values were plotted (graph not shown), they fitted exponential curves that only differed in OP for the highest RH with a sharp increase at 23 °C. The increase in OP with increasing temperature was expected and correlates with Arrhenius equation (Selke and Culter 2016). This exponential effect of increasing RH on OP has also been observed for protein-based edible films (Gontard et al. 1996). The increase in OP as the RH increases is explainable by the loss of glycerol with increasing RH. Lower glycerol content resulted in more water diffusing through the polymer matrix. This resulted in more space between polymer chains that increased the oxygen diffusion through the polymer matrix. In addition, the higher water content in the film increased the solubility of oxygen in the film. Oxygen solubility in water has been reported to be 6.0 mL L^{-1} at 23 °C and 1 atm (Weiss 1970). The increased oxygen diffusion and solubility rates with the increase in RH led to higher OP values. The OP of the EWP film at 0% RH and 23 °C was 0.41×10^{-18} kg m m^{-2} s^{-1} Pa^{-1}, which is one order of magnitude lower than the OP of the PLA film under the same conditions (5.67×10^{-18} kg m m^{-2} s^{-1} Pa^{-1}) (González-Buesa et al. 2014). This finding suggests that the EWP film would work better than the PLA film for food packaging applications where protection to avoid oxidation is needed. Specifically, EWP film applications should focus on packaging for food products with a much higher sensitivity to oxygen than water.

The EtOhP of EWP film ranged between 3.6×10^{-17} and 14.5×10^{-17} kg m m^{-2} s^{-1} Pa^{-1} (Table 4). This value is in the same range as that of LDPE (3.2×10^{-17} kg m m^{-2} s^{-1} Pa^{-1} (Robertson 2013)) and a little bit higher than that of PLA (2.0×10^{-17} kg m m^{-2} s^{-1} Pa^{-1} (González-Buesa et al. 2014)). This finding indicates that the EWP film would work similarly to both LDPE and PLA in terms of retaining ethanol levels inside a package. There was an effect of both temperature and RH on EtOhP (Table 3). The EWP films stored at 4 °C and 23 °C showed an increase in EtOHP with an increase in RH up to values of 55% and 95% RH, respectively. This can be explained by the increase in water content in the films during

Table 4 EWP and PLA permeability values for water, oxygen, and ethanol after exposure to different conditioning treatments (N/A means "not applicable")

Film	Temperature (°C)	RH (%)	Permeability (kg m m^{-2} s^{-1} Pa^{-1})		
			Water (× 10^{-12})	O_2 (× 10^{-18})	Ethanol (× 10^{-17})
EWP	4[1]	0	N/A[2]	0.35 ± 0.14^{Aa}	7.97 ± 2.84^{Aa}
		55	0.28 ± 0.11^{Aa}	1.60 ± 0.49^{ABa}	12.23 ± 1.38^{Ba}
		95	3.85 ± 0.37^{Ba}	5.97 ± 3.06^{Ba}	12.58 ± 1.04^{Ba}
	23	0	N/A	0.41 ± 0.23^{Aa}	3.56 ± 0.71^{Ab}
		55	0.46 ± 0.05^{Aa}	2.39 ± 0.08^{Aa}	5.79 ± 0.23^{Ab}
		95	2.15 ± 0.88^{Bb}	40.23 ± 6.24^{Bb}	14.50 ± 0.04^{Ba}
PLA[3]	23	55	Not reported	5.67	2.0
		95	0.02	Not reported	Not reported

[1] Water and O_2 permeability were measured at 10 °C instead of 4 °C due to equipment limitations

[2] Water permeability was not measured at 0% RH due to equipment limitations

[3] Permeability values reported by González-Buesa et al. 2014

conditioning, caused by the loss of glycerol with increasing RH, that affected the ethanol solubility and diffusion rates in a manner similar to the way oxygen was affected. Ethanol is soluble in water because the polarity of its hydroxyl group is stronger than the non-polarity of its 2-carbon chain. Lower EtOHP was observed in EWP films at higher temperatures except for those at 95% RH due to an interaction between temperature and RH ($P = 0.005$; Table 3). This interaction can be explained by the increase in water content in the films stored at 95% RH and 23 °C compared to 95% RH and 4 °C due to the effect of temperature on water vapor pressure, which subsequently affected both the diffusion and solubility of ethanol as discussed above. The lower EtOHP at higher temperatures can be explained by the uptake of more water at lower temperatures at constant RH that characterizes most foods and biomaterials (Bell and Labuza 2000). Similarly, temperature and RH had significant effects on the permeability of the aroma compound *d*-limonene in whey protein isolate film (Miller et al. 1998).

Optical Properties

The color (a^* and b^*) of the EWP films was affected by RH ($P = 0.000$; Table 3), but not by temperature ($P > 0.05$; Table 3). The EWP films stored at 0% RH and 95% RH had a same color that was less reddish and more yellowish compared to the films stored at 55% RH for both temperatures (Table 5). In contrast, the lightness (L^*) of the EWP films was affected by temperature ($P = 0.049$; Table 3) but not RH ($P > 0.05$; Table 3). The EWP films at 55% RH became darker when stored at a higher temperature compared to the EWP films exposed to lower and higher RH due to an interaction between temperature and RH ($P = 0.047$; Table 3). The authors have no explanation for the color and lightness changes observed at 55% RH. When comparing our results to published information pertaining L^*, a^*, and b^* of compressed EWP bioplastic (Lee et al. 2013), the EWP films are lighter (91.5% compared to 87%), more reddish (− 1.45 compared to − 2.4), and less yellowish (2.7 compared to 17) than compressed EWP bioplastic. The difference in color is most likely due to the different thicknesses between our EWP films and

Table 5 Optical properties (color and transmittance) of EWP films after exposure to different conditioning treatments and of PLA film after exposure to 55% RH and 23 °C

Film	Temperature (°C)	RH (%)	Color			Transmittance (%)
			L^*	a^*	b^*	
EWP	4	0	91.7 ± 0.3^{Aa1}	-1.46 ± 0.14^{Aa}	2.47 ± 0.51^{ABa}	91.5 ± 2.0^{Ba}
		55	91.9 ± 0.4^{Aa}	-1.32 ± 0.08^{Ba}	2.00 ± 0.40^{Ba}	94.4 ± 0.9^{Aa}
		95	91.8 ± 0.3^{Aa}	-1.55 ± 0.13^{Aa}	2.94 ± 0.80^{Aa}	93.6 ± 1.3^{Aa}
	23	0	91.6 ± 0.6^{ABa}	-1.47 ± 0.21^{Aa}	2.76 ± 0.98^{Aa}	93.7 ± 1.6^{Bb}
		55	91.5 ± 0.4^{Ab}	-1.30 ± 0.13^{Ba}	2.11 ± 0.63^{Ba}	94.2 ± 0.7^{ABa}
		95	91.8 ± 0.3^{Ba}	-1.49 ± 0.18^{Aa}	2.74 ± 0.74^{Aa}	94.9 ± 0.8^{Ab}
PLA	23	55	92.9 ± 0.1	-0.07 ± 0.01	1.54 ± 0.04	95.5 ± 0.1

[1] Different uppercase letters and lowercase letters indicate significant differences ($P \leq 0.05$) caused by RH and temperature, respectively

the compressed EWP bioplastics. In agreement with the above results, Lee et al. (2013) reported that the thinner the compressed EWP sheet is, the lighter and the less green and yellow it becomes. Similarly, Gennadios et al. (1996) indicated that thicker soy-based films were more yellowish than thinner ones. Comparing the color of EWP films with that of PLA stored under the same conditions (23 °C and 55% RH), both bio-based films have similar lightness (91.8 vs. 92.9) and b^* values (2.1 vs. 1.5) but not a^* values (− 1.30 vs. − 0.07). Joo et al. (2011) reported similar lightness (92.5) and color (1.6 and − 0.11) value for PLA films obtained by the extrusion and calendering processes and stored at 23 °C and 55% RH. Based on obtained and reported values for PLA films, the EWP film is a little bit more greenish than the PLA film. This should not be a problem from the consumer point of view since there are several commercially available plastics with a green color tinge that are used for food packaging applications like polyvinyl chloride and polyvinylidene chloride copolymers (Selke and Culter 2016).

As observed in Table 5, EWP films obtained by extrusion and calendering processes are highly transparent (92 to 95% at 600 nm wavelength). These films are more transparent than other EWP bioplastics reported in the literature [38% (González-Gutiérrez et al. 2010) and 89% (Lee et al. 2013), both at 600 nm]. The improvement in EWP bioplastic transparency may be attributed to the higher orientation of the polymer chains caused by the extrusion and calendering processes compared to the compression process. The considerable difference in transparency between our films and those obtained by González-Gutiérrez et al. (2010) may be explained by their different composition (plain EWP vs. a mixture of EWP and starch, respectively). It should be noted that EWP films obtained by extrusion and calendering processes in the present study are as transparent as commercially available PLA film conditioned under the same conditions (55% RH and 23 °C) (Table 5). Both temperature and RH had an effect on the transmittance of light through the EWP films ($P = 0.000$; Table 3). The transmittance values of the EWP films stored at 23 °C were higher than those of the EWP films stored at 4 °C, except for the EWP films exposed to 55% RH (interaction temperature and RH; $P = 0.003$; Table 3). The difference in transmittance caused by temperature can be attributed to the exposure of the EWP films to a different amount of water molecules as the vapor pressure of the water increases with increasing temperature (Kessler 2006). The more water molecules surrounding the EWP film resulted in more evaporation of glycerol and water from the film due to the higher loss of the two molecules with increasing surrounding water content as reported by the Glycerine Producers' Association (1963). The evaporation of these two film components may have led to a tighter polymer matrix that allowed for more light to go through it. The decrease in RH at both studied temperatures resulted in significant decreases in the transmittance values of the EWP films significantly ($P \leq 0.05$; Table 5). This was due to the differences in film thickness caused by RH. As reported in the "Thickness" section, the thickness of the EWP film decreased with an increase in RH due to the loss of glycerol. This loss of glycerol with increasing RH may have resulted in a tighter polymer matrix that allowed for more light to transmit through the film.

Fungal Resistance

The EWP film did not pass the ASTM fungal resistance testing since the film did not avoid the growth of *P. pinophilum* and *A. niger* for 21 days (Fig. 4). However, it needs to be taken into consideration that the ASTM method G21 was developed for petroleum-based plastics, which do not interact with the agar of the petri dish as the EWP film does. This interaction may have resulted in the migration of agar nutrients from the agar into the EWP film, which fed the fungi. In addition, the testing was performed at 100% RH instead of 85% RH, which

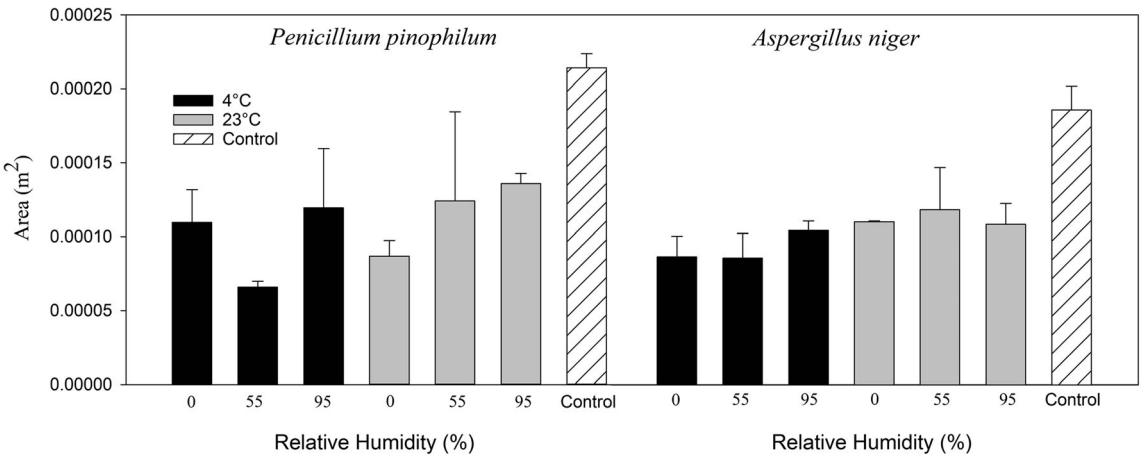

Fig. 4 Growth of *P. pinophilum* and *A. niger* on EWP films exposed to different conditioning treatments compared to controls after 48 h incubation at 23 °C and 100% RH

substantially increases fungal growth. The closing of the petri dishes with their lids created the higher RH environment. These results show that a fungal resistance testing different from the ASTM method G21 is necessary for bio-based materials. In this regard, new methods have been developed (Le Bayon et al. 2015; Palumbo et al. 2017); however, there is not yet a standardized/recognized method to assess fungal resistance of bio-based materials. This method will most likely have to consider real storage conditions, adequate fungal strains, and possible migrating nutrients if any.

The results show that the EWP film was able to reduce the growth of both *A. niger* and *P. pinophilum* by half at 100% RH and 23 °C compared to the control (no EWP film). The average growth area of *P. pinophilum* was 1.1×10^{-4} m^2 on the films compared to 2.1×10^{-4} m^2 on the agar. Similarly, the EWP film reduced *A. niger* growth (1.0×10^{-4} m^2 on the films compared to 1.9×10^{-4} m^2 on the agar). Most likely, the film did not allow the diffusion of all the nutrients from the agar. Alternatively, non-polymerized proteins could have affected the growth of the fungi if they did not denature during film processing. Some of the main egg white proteins have demonstrated antimicrobial activities (Kovacs-Nolan et al. 2005; López-Mata et al. 2016) and consequently, these have been used to develop antimicrobial films (Corradini et al. 2013; López-Mata et al. 2016). Comparing the different treatments, neither temperature nor RH had an effect on fungal growth except for temperature on *A. niger* growth for which increased temperature was associated with increased growth ($P = 0.031$; Table 3). This could be due to the interaction of the films with the agar, which changed the properties of the films and made them similar overtime. The antifungal testing results indicate that the EWP film seems to be a good choice for the packaging of dry food products but not of moist food products. This is because dry food products are unlikely to generate high RH and consequently, nutrients would be unlikely to migrate into the EWP film. However, the EWP film may be a desired material to package moist food products if it possesses antimicrobial activity, and this warrants further testing.

Conclusions

The appropriate extrusion and calendering conditions for the development of EWP films for industry adaptation have been determined. These conditions result in a transparent, continuous, thin, and uniform film that can be used for specific food packaging applications. This potential is evidenced by the film performance when exposed to specific combinations of RH and temperature that mimic the storage conditions of a variety of food products and by its performance when compared with PLA, the most used bio-based material for food packaging applications. Specifically, the low loss of glycerol content during storage, especially at lower RH, indicates that the EWP film is a viable alternative to other packaging materials for semi-solid and solid food products, especially for those with a low water activity. The thermal resistance of approx. 150 °C makes EWP film suitable as a packaging material for food treatments like sterilization, processing, and cooking up to that temperature and for food treatments unfit for PLA like microwaving. The mechanical properties of the EWP film are most appropriate for food packaging when rigidity and strength are desired. EWP film applications should focus on packaging for food products with a much higher sensitivity to oxygen than to water since the film presents a very good barrier to oxygen but not to water. Similarities between the EWP film and the PLA film in terms of lightness, color, and transmittance suggest consumer acceptability of the former. Fungal growth occurs when the EWP film is contaminated with spores in in vitro studies. This indicates that it is not an optimal material to package moist food products, but it is most likely a good choice for dry food products. This study proves the feasibility of producing EWP films using the most common filmmaking processing technology and shows that the EWP film can be used for specific food packaging applications. Further research is needed to improve the water barrier, fungal resistance, and mechanical properties of the EWP film so it can perform at a wider range of conditions, which in turn will expand its applicability for food packaging. However, legal aspects need to be taken into account.

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