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Investigation on the film-forming properties of lab fermenter scale produced scleroglucans from *Sclerotium rolfsii* ATCC 201126

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ABSTRACT

The film-forming capacity of the biopolymer scleroglucan produced on the lab fermenter scale by *Sclerotium rolfsii* ATCC 201126 was studied. The effect of a freeze–thawing process on the physical properties of scleroglucan films was investigated with hydrogels of three different scleroglucan samples. Films were made from 1% (w/w) scleroglucan hydrogels using 2% (w/w) glycerol as plasticizer by applying two different protocols: (1) a room-temperature drying method and, (2) a freeze–thawing cyclic process (prior to the application of room-temperature drying). The obtained materials were characterized by physical studies including swelling, water vapor transmission, and environmental scanning electron microscopy. Based on the results obtained, when eight freezing–thawing cycles were applied during scleroglucan film preparation, a reinforcement of the structure was achieved pointing to an increase in the number of crosslinking points by H-bonding. All exopolysaccharides produced by *S. rolfsii* ATCC 201126 at lab fermenter scale showed a general promising behavior for the preparation of reinforced films, opening new perspectives for their potential use in either controlled release systems or the formulation of additive-complemented films for specific applications.

1. Introduction

Hydrophilic neutral β-1,3-β-1,6-glucans commonly called 'scleroglucans' are polysaccharides produced by filamentous fungal species of the genus *Sclerotium*. Among them, the biopolymer excreted to the culture medium by *Sclerotium rolfsii* has been the focus of recent investigations as a hydrophilic polymer with great ability to form three-dimensional network structures or gel structures, even at low polymer concentrations (Viñarta, François, Daraio, Figueroa, & Fariña, 2007; Zupanèiè, 1998). Particular features of scleroglucan such as water solubility, biocompatibility, resistance to hydrolysis as well as the viscosifying ability even at high temperature (100 °C, 60 min), high ionic strength (up to 20% (w/v) NaCl) and over a wide range of pH (0–13) (Fariña, Siñeriz, Molina, & Perotti, 2001) make it attractive for a diversity of industrial applications.

Based on above mentioned attributes, the use of scleroglucan has been considered for enhanced oil recovery (EOR), applications in paper processing paints, cosmetics and pharmaceutical products, quality improvement of foods, immune stimulating effects, and controlled drug delivery (Coviello et al., 2005; François, Rojas, Daraio, & Bernik, 2003; François, Rojas, & Daraio, 2005; Kulicke, Lettau, & Thielking, 1997; Survase, Saudagar, Bajaj, & Singhal, 2007; Viñarta, Molina, Figueroa, & Fariña, 2006; Viñarta et al., 2007).

As already mentioned, scleroglucan can be produced by different *Sclerotium* species, and variances have been frequently reported concerning molecular weight, number and length of side chains, degree of polymerization, degree of β-1,6-glycosidic branching and rheological characteristics depending on the species, strain, culture conditions or even the downstream processing (Bluhm, Deslandes, Marchessault, Pérez, & Rinaudo, 1982; Singh, Whistler, Tokuzen, & Nakahara, 1974; Sletmoen & Stokke, 2008; Zentz & Muller, 1992). According to the literature, fundamental studies have mainly focused on scleroglucan from *S. glucanicum* has although scleroglucan produced by *S. rolfsii* may become attractive from a commercial point of view (Bluhm et al., 1982; Wang & McNeil, 1996). However, few investigations have dealt with *S. rolfsii* scleroglucan produced on a lab fermenter scale. Commercially available scleroglucans are the ones most frequently evaluated.

In many fields of application, such as transdermal drug delivery (Nicoli, Colombo, & Santi, 2005), coatings for food protection (Alves et al., 2011; Flores, Famá, Rojas, Goyanes, & Gerschenson, 2007; Tharanathan, 2003), and materials for wound healing (Schmidt, 2005), films obtained from biocompatible polymers play an important role.

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Although different commercially available scleroglucans produced on the industrial level have been investigated for their application in drug delivery systems (Coviello et al., 2003; François et al., 2003, 2005; François & Daraio, 2009), literature becomes scarce concerning the use of lab-scale or pilot-plant produced scleroglucans from recently isolated *Sclerotium* strains (Viñarta et al., 2007). Taking into account the advances on the knowledge of the physicochemical properties of *S. rolfsii* ATCC 201126 scleroglucan (Fariña et al., 2001; Fariña, Viñarta, Cattaneo, & Figueroa, 2009), the film-forming ability of different lab fermenter scale produced polysaccharides was examined based on different room-temperature and freeze–thawing protocols for preparation.

2. Materials and methods

2.1. Scleroglucan samples

Scleroglucan tested samples consisted in three exopolysaccharides (EPSs) from *S. rolfsii* ATCC 201126, namely EPS I, EPS II and EPSi, produced under batch culture mode at fermenter scale. In the three cases, the Czapek malt activated strain was first seeded in PM_{20} liquid medium (Fariña, Siñeriz, Molina, & Perotti, 1998) and the resulting inocula were then blended under aseptic conditions (CB-6 Waring blender, minimum output) and used at 10% (v/v) at fermenter scale. An identical stirred-tank reactor design fitted with baffles and six-flat bladed Rushton turbine impellers was used for the production of all EPSs, and the only difference was a working volume of 8 L for EPS I and EPS II (MicroFerm, New Brunswick Scientific Co.), and a working volume of 2 L for EPSi (LH 210 Series, Inceltech). The optimized culture medium (MOPT) for polysaccharide production contained (in $g\,L^{-1}$): sucrose, 150; $NaNO_3$, 2.25; $K_2HPO_4{\cdot}3H_2O$, 2; yeast extract, 1; citric acid$\cdot H_2O$, 0.7; KCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; $FeSO_4{\cdot}7H_2O$, 0.05 (initial pH 4.5). The following conditions were maintained throughout the three fermentations: air flow rate, 0.5 vvm; stirrer speed, 400 rpm; temperature, 30 °C, pH uncontrolled.

EPS I and EPS II were recovered at two different fermentation times (48 and 72 h, respectively) and ethanol-purified, as previously described (Fariña et al., 2001). On the other hand, EPSi was recovered at 72 h of cultivation, and downstream processed with isopropanol. In the three cases, downstream processing included a three-fold dilution of culture broth with distilled water, neutralization and homogenization (CB-6 Waring blender, minimum output), followed by heating at 80 °C for 30 min. After centrifugation (10,000 × *g*, 30 min, 20 °C), the EPS from clear supernatant was cooled at 5 °C and precipitated by adding an equivalent volume of the corresponding alcohol. This mixture was allowed to stand at 5 °C for 8 h to complete EPS precipitation. The precipitate was recovered with a fine sieve (Macotest ASTM No. 60) and then redissolved in distilled water. This crude EPS was further purified by alcohol-reprecipitation (two times).

All precipitated EPSs were finally freeze-dried and milled to a whitish glucan powder, giving different purified preparations of scleroglucan. Glucan powder was analyzed for protein content by the Folin–Lowry method using bovine serum albumin as standard. Reducing sugars were measured according to the Somogyi–Nelson method (Hodge & Hofreiter, 1962) with glucose as standard. Total carbohydrates were determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) with dextran as standard. EPSs M_w was determined by size exclusion chromatography coupled to laser light scattering (SEC-LLS), as follows. EPS was dissolved in the mobile phase in order to obtain a 2 $mg\,mL^{-1}$ final concentration. Samples (200 μL) were injected in duplicate into a Waters-Breeze liquid chromatograph with an automatic injector (Waters 717 plus) coupled to a differential refractometer (Waters 2414, model 410) and a light scattering detector (Dawn DSP). Size exclusion chromatography (SEC) involved the use of an Ultrahydrogel 5-column set (Waters, 7.8 mm × 300 mm; pore sizes: 120, 250, 500, 1000, 2000 Å). The mobile phase consisted in $NaNO_3$ (0.1 M) plus NaN_3 (0.02%, w/v) and flow rate was set at 0.8 $mL\,min^{-1}$.

The three purified EPSs, i.e. EPS I, EPS II and EPSi, were evaluated on their film-forming ability. Scleroglucan gels were used at 1% (w/w) in all film preparations. Glycerol analytical grade (Cicarelli, Argentina) was used at 2% (w/w) as plasticizer to improve the physical properties of the films by decreasing their brittleness. Water was purified using a Millipore Simplicity System.

2.2. Preparation of scleroglucan films

2.2.1. Room temperature drying (RTD)

Scleroglucan hydrogels were prepared dispersing the required amount of polymer powder in purified water plus glycerol. Dispersions contained in sealed small glass beakers, to avoid water loss by evaporation, were kept at constant temperature of (25 ± 1) °C under magnetic stirring for 96 h in order to obtain complete swelling and homogeneous gel formation. Well-defined stirring conditions were kept constant for each gel preparation.

After obtaining the hydrogel, a certain amount was poured and spread onto a leveled, circular (5-cm diameter) polypropylene plate. The material was initially dried at 50 °C during 1 h and then, allowed to dry at room temperature in contact with ambient air for a week until semi-transparent films were obtained.

2.2.2. Freeze–thaw (FT) cycling

Hydrogels prepared as described in Section 2.2.1 were poured onto the polypropylene plates and placed at −20 °C for 24 h. After the freezing process, they were thawed at room temperature (~25 °C) for 1 h. This freezing and thawing cycle was repeated eight times. Finally, the material was dried at 50 °C during 1 h and allowed to dry at room temperature for a week, as above.

2.3. Swelling assays

For these experiments, the film sample contained in a stainless steel basket was subjected to total immersion in purified water at 25 °C. The absorbed amount of water was determined by weighting, after wiping, at various time intervals. Swollen films were weighted with an electronic balance (ACCULAB, capacity 210 g) to the nearest 0·1 mg.

Films were characterized by the swelling degree's variation in time (Q_t) determined as:

$$Q_t = \frac{(m_t - m_0)}{m_0} \quad (1)$$

where, Q_t is the swelling degree at time t, m_t is the mass of the swollen film at time t, m_0 is the mass of the dry sample at time 0 and $(m_t - m_0)$ is the weight of the solvent absorbed by the film at time t.

The kinetics of water uptake (M_t/M_∞) (Peppas & Franson, 1983) was analyzed using the following empirical equation:

$$\frac{M_t}{M_\infty} = k' t^n \quad (2)$$

where M_t is the mass of water absorbed at time t, M_∞ is the mass of water absorbed at equilibrium, k' (min^{-n}) is a typical constant of the hydrogel, and n is the characteristic exponent describing the mechanism of water uptake.

Eqs. (1) and (2) lead to:

$$\frac{Q_t}{Q_\infty} = k' t^n \quad (3)$$

where Q_t is the swelling degree at time t.

Then, Q_∞ and k' were included in k, and the following equation was used to fit the swelling data:

$$Q_t = kt^n \quad (4)$$

2.4. Water vapor transmission (WVT) and water vapor permeability (WVP) measurements

Acrylic-made water vapor permeation cells with an internal diameter of 5.0 cm and an external diameter of 8.5 cm were used. The film was fixed with a 4-screw fastened acrylic ring-shaped cover. Cells were 3.5 cm in depth and contained $CaCl_2$ (0% relative humidity (RH), 0 Pa water vapor partial pressure).

The WVT rate through the films was determined using a modified ASTM E96-00 method (ASTM, 2000). To do this, a film sample was placed between the water vapor permeation cell and its acrylic ring-shaped cover. A 10-mm air gap was left between the film and the $CaCl_2$ layer. The cells were stored in an isolated chamber at a constant temperature of 28 °C and a RH of 71%, which were systematically weighted once a day.

Water vapor transport was determined from the weight gain of the cell. All tests were conducted in duplicate.

The water vapor transmission rate (WVT, $g\,m^{-2}\,s^{-1}$) is the steady water vapor flow per unit time through unit area of a body, normal to specific parallel surfaces, under specific conditions of temperature and humidity at each surface. Enough time (24 h) was allowed between measurements to ensure a steady-state in WVT rate measurements. Changes in weight were daily recorded (at the nearest 0.1 mg) over an 8-day period. The weight gain of permeation cells as a function of time showed linear behavior and the slope of each curve was calculated by linear regression. WVT rate was calculated from the slope of the straight line dividing by the exposed area of the film ($7.07 \times 10^{-4}\,m^2$).

Permeance ($g\,m^{-2}\,s^{-1}\,Pa^{-1}$) is the time rate of water vapor transmission through unit area induced by unit vapor pressure difference between two specific surfaces, under specified temperature and humidity conditions.

Permeance was calculated as:

$$\text{Permeance} = \frac{\text{WVT}}{S^*(R_e - R_p)} \quad (5)$$

Where S is the saturation vapor pressure of water at test temperature (3649.56 Pa in our experiments), R_e is the RH at the test chamber (71%) expressed as a fraction and R_p is the RH inside the permeation cell (0%) expressed as a fraction.

The water vapor permeability is the time rate of water vapor transmission through unit area of flat material of unit thickness induced by unit vapor pressure difference between two specific surfaces, under specified temperature and humidity conditions. It is the arithmetic product of permeance and thickness.

The thickness of films was measured at five different positions and to the nearest 0.001 mm using a digital thicknessmeter (Schwyz, type II) with 10 mm diameter ceramic contact faces.

2.5. Environmental Scanning Electronic Microscopy (ESEM)

Micrographs of scleroglucan films with and without freezing–thawing cycles were obtained at 20 °C using an Environmental Scanning Electron Microscope 2010 (FEI Company, Hillsboro, OR) running in the so-called environmental wet mode. The sample chamber constant pressure and the electron beam voltage are displayed at the bottom of each micrograph.

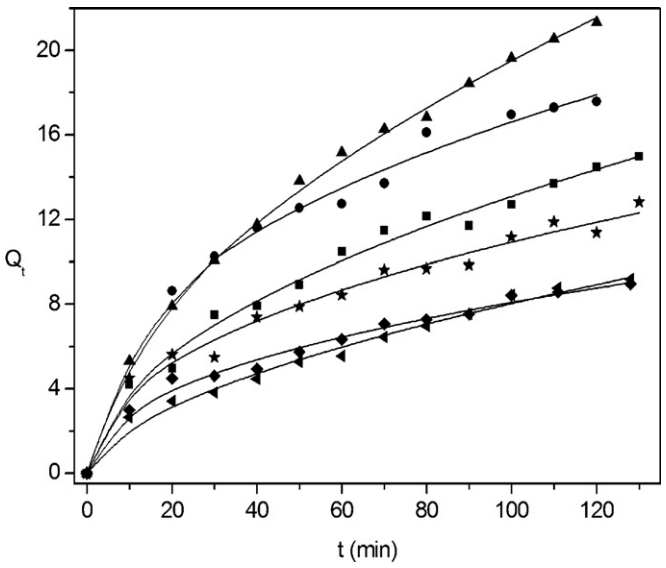

Fig. 1. Effect of 8 freezing and thawing (FT) cycles on the swelling curves of films obtained from different *S. rolfsii* ATCC 201126 scleroglucans. EPS I: (■) $n_{FT} = 0$, (⋆) $n_{FT} = 8$; EPS II: (▲) $n_{FT} = 0$, (●) $n_{FT} = 8$; EPSi: (♦) $n_{FT} = 0$, (◄) $n_{FT} = 8$.

3. Results and discussion

3.1. Swelling assays

Swelling measurements are employed as a rather simple method to characterize polymer networks. A lowering in the swelling degree can be helpful to deduce the existence of a higher number of crosslinking points in physical gels (Chen, Chen, Lu, Bu, & Yang, 2003). The phenomenon of solvent sorption by a polymeric film mechanistically depends on the diffusion of water molecules into the gel matrix and the subsequent relaxation of macromolecular chains (Bajpai & Sharma, 2005). Since the degree of hydration is one of the factors determining the velocity of drug release from hydrogel matrices, the swelling kinetics as described by the water penetration parameters, represents an important characterization in materials tested for drug delivery applications (Colombo, Bettini, Santi, De Ascentiis, & Peppas, 1996).

The effect of both preparation methods (RTD and FT) on the swelling behavior of scleroglucan films is shown in Fig. 1. A similar trend was observed for the different EPSs tested. As a consequence of the applied FT cycles, the water uptake became reduced, predominantly in EPS I and EPS II. This result suggested that FT cycles promoted the increase in the number of crosslinking sites, thus hindering the solvent diffusion into the films and thereby decreasing the degree of swelling. This fact suggests the existence of a denser structure, very likely due to an increase in the physical crosslinking points in EPS I and EPS II networks.

Although the swelling degree Q_t was different among tested EPSs (Fig. 1), according to Table 1, the swelling exponent n was near 0.5 for all samples. For a given film, when the exponent of Eq. (4) is $n = 0.5$, it indicates Fickian diffusion, $0.5 < n < 1$ means non-Fickian or anomalous transport, and $n = 1$ implies case II (relaxation controlled) transport (Peppas & Khare, 1993). The n exponents in Table 1 indicate that the water uptake process in every tested scleroglucan film is kinetically controlled by the diffusion of water into the polymeric structure.

The swelling behavior, other physical characteristics and the drug delivery pattern of films from a commercial scleroglucan (LSCL) were presented in a previous study (François & Daraio, 2009). In that case, for a film obtained by RTD method, a Q_t swelling degree of 13.4 was obtained at 100 min interval, whilst by applying 8 FT

Table 1
Swelling kinetic parameters[a] obtained for *S. rolfsii* ATCC 201126 scleroglucan films prepared at different conditions.

EPSs	k (min^{-n})[b]	n[b]	R^2
EPS I – RTD	1.2 ± 0.3	0.51 ± 0.05	0.9904
EPS I – FT	1.4 ± 0.4	0.45 ± 0.06	0.9833
EPS II – RTD	1.6 ± 0.2	0.55 ± 0.02	0.9984
EPS II – FT	2.5 ± 0.6	0.41 ± 0.05	0.9919
EPSi – RTD	1.1 ± 0.2	0.44 ± 0.05	0.9902
EPSi – FT	0.6 ± 0.1	0.58 ± 0.06	0.9898

RTD: Films prepared under room temperature drying.
FT: Films prepared with eight freezing and thawing cycles.
[a] Mean and confidence intervals are informed. All parameters are within the 95% confidence interval of the nonlinear least square estimate.
[b] Kinetic parameters obtained by fitting the data through Eq. (4).

cycles Q_t was 8.4. These results would be comparable to those herein obtained for EPS I (Fig. 1), whilst EPS II showed a better swelling behavior. This was not surprising since previous studies concerning the dynamic rheological behavior and the hydrogel microstructural properties also found similarities between EPS I and LSCL commercial scleroglucan, at difference with EPS II (Viñarta et al., 2007).

Several properties previously investigated for *S. rolfsii* ATCC 201126 scleroglucan aqueous solutions, such as viscosifying ability and stability of apparent viscosity against high temperatures revealed a better performance of EPS II *vs*. EPS I (Fariña et al., 2001). Similarly, the capacity to prevent syneresis in corn starch pastes, as well as the suspending, dispersing and emulsifying properties showed better results with EPS II than with EPS I (Viñarta et al., 2006, 2007). Meanwhile, EPS behavior in the presence of different inorganic salts or high NaCl concentrations, as judged by apparent viscosity measurements, resulted more stable in the case of EPS I than for EPS II (Fariña et al., 2001).

On the other hand, when drug release patterns were evaluated for *S. rolfsii* ATCC 201126 EPSs, almost no differences could be found between EPS I and EPS II, independently of the M_w or the microstructural properties of the scleroglucan gel matrices (Viñarta et al., 2007). Drug delivery assays demonstrated that even hydrogels with different pore sizes (7 μm for EPS II, and 20 μm for EPS I), led to quite similar theophylline release kinetics, being polymer concentration probably the main factor influencing drug release (Viñarta et al., 2007).

It is suspected that different polysaccharide characteristics govern these differential behaviors. In the case of EPS solutions, a viscosity decrease in the presence of salts was previously related to the solvent quality reduction and the increase in intramolecular interactions which may lead to coil contraction (Fariña et al., 2001). In this sense, conformational differences such as the coiling degree, may account for the higher viscosity drop of EPS II than EPS I aqueous solutions when confronted to high NaCl concentrations (Fariña et al., 2001). In EPS II, a more expanded coil may exhibit higher rigidity causing higher viscosity but, when exposed to osmotically active molecules which can compromise the solvent, this macromolecular structure may be more sensitive and consequently, the reduction in viscosity may be much more pronounced.

This higher interaction of EPS II with the solvent (water) may also explain in the present study, why this scleroglucan preparation exhibited a better swelling behavior. However, when exposed to FT cycling, this polysaccharide experienced a similar Q_t reduction as EPS I (Fig. 1). In this sense, it may be interesting to highlight the similarity in Q_t reduction between EPS I and EPS II, both recovered and purified with ethanol whilst EPSi, purified by the isopropanol protocol, showed almost identical swelling behavior in RTD and FT films (Fig. 1).

Despite the fact that crosslinking magnitude may increase with the number of FT cycles, it has been observed for other polymer systems that swelling was more influenced by the overall hydrophilicity of the sample than for the number of crosslinks (Brazel & Peppas, 1999). This rationale may also be applied to explain why EPS II, which has been correlated to hydrogels with a higher crosslinking (Viñarta et al., 2007), formed films more swellable than EPS I (Fig. 1).

A further explanation for interpreting swelling behavior would be related to the polymer M_w (Brazel & Peppas, 1999). Laser light scattering measurements indicated that EPSi would exhibit a slightly higher M_w (2.7×10^6 Da) than EPS I and EPS II ($\sim 1 \times 10^6$ Da). The great number of intermolecular bonds could then lead to a more closed network structure, thus explaining why films formed from EPSi would swell to a lower degree than similar films prepared from the other EPSs.

Considering the high similarity in EPS production (volumetric productivity ~0.3–0.36 g/L h) and purity parameters (recovery yield, EPS composition – Table 2), and that nutritional and operative conditions were kept the same for the three fermentations, we suspect that the influence on the EPSi differential swelling behavior would be more related to its downstream processing with isopropanol than to the change in the fermenter working volume.

A different precipitation protocol might have influenced the hydrophilicity of the sample or favored the recovery of macromolecules with a slightly higher average M_w (Viñarta, 2009). Differences in film behavior of commercial scleroglucan might be analyzed under a similar rationale, since it has been already mentioned that scleroglucan properties may be influenced by the molecular mass and by recovery methods. Taking into account that M_w and polydispersity could be stable under different fermentation conditions (Survase et al., 2007), a major source of variation might be thus expected from downstream processing. As already emphasized, solution properties of scleroglucan may be different, depending on the polymer grade used (Survase et al., 2007).

Regardless of these minor differences, the possibility to obtain reinforced FT scleroglucan films without significantly affecting the swelling behavior could be confirmed.

3.2. WVT and WVP measurements

From the weight gain of the permeation cells as a function of time, the slope of each curve was calculated by linear regression obtaining a correlation coefficient R^2 over 0.998 in every case.

WVT and WVP data for scleroglucan films obtained according to both methods are shown in Table 3. Results indicated that only in the case of EPS I the method of film preparation minimally affected WVT and WVP when 8 FT cycles were applied, but not for EPS II and EPSi. Considering previous WVT data for a commercial scleroglucan (LSCL), i.e. $1.38 \pm 0.04 \times 10^{-2}$ g s^{-1} m^{-2} and $1.24 \pm 0.01 \times 10^{-2}$ g s^{-1} m^{-2} for RTD and FT films, respectively (François & Daraio, 2009), this observation would be in agreement with swelling assays, which showed a more similar behavior between EPS I and commercial scleroglucan. In the case of scleroglucan films, the rather high transmission of water vapor and permeability are favored due to the large number of hydrophilic groups in the biopolymer structure. It is important to remark that the FT method of film preparation only decreased the moisture permeability by no more than 10% whilst resulting in a reinforced physically crosslinked network, as indicated by swelling experiments.

Again, although some differences in water vapor permeability were observed, results demonstrated that all scleroglucan films from *S. rolfsii* ATCC 201126 exhibited a similar performance. This finding would highlight the convenience to obtain these films by FT

Table 2
Recovery yield and purity grade parameters for scleroglucan samples recovered from different *S. rolfsii* ATCC 201126 culture broths after different post-fermentation processing.

EPSs[a]	Recovery yield (%)[b]	Moisture content (%, w/w)	Proteins (%, w/w)	Total sugars (%, w/w)	Reducing sugars (%, w/w)
EPS I	16.1	12.56	1.9	98.0	0.1
EPS II	18.4	13.54	1.6	98.0	0.4
EPSi	22.9	2.96	1.45	87.9	1.8

[a] EPS I and EPS II were recovered and purified by ethanol precipitation at 48 and 72 h of fermentation, respectively. EPSi was recovered and purified by isopropanol precipitation at 72 h of fermentation.
[b] Estimate referred on a dry basis.

Table 3
Water vapor transmission (WVT; $T = 25\,^{\circ}C$; % RH = 71) and water vapor permeability (WVP) for *S. rolfsii* ATCC 201126 scleroglucan films obtained by either room temperature drying (RTD) or freeze–thawing (FT) methods.

EPSs	WVT ($g\,s^{-1}\,m^{-2}$)		WVP ($g\,m^{-1}\,s^{-1}\,Pa^{-1}$)	
	RTD	FT[a]	RTD	FT[a]
EPS I	$(1.21 \pm 0.03) \times 10^{-2}$	$(1.14 \pm 0.02) \times 10^{-2}$	$(4.20 \pm 0.10) \times 10^{-10}$	$(3.96 \pm 0.07) \times 10^{-10}$
EPS II	$(1.22 \pm 0.03) \times 10^{-2}$	$(1.20 \pm 0.02) \times 10^{-2}$	$(4.23 \pm 0.10) \times 10^{-10}$	$(4.17 \pm 0.07) \times 10^{-10}$
EPSi	$(1.24 \pm 0.03) \times 10^{-2}$	$(1.20 \pm 0.01) \times 10^{-2}$	$(4.31 \pm 0.10) \times 10^{-10}$	$(4.17 \pm 0.03) \times 10^{-10}$

The average thickness of films obtained by RTD and FT methods is 0.090 ± 0.01 mm.
[a] With 8 cycles.

cycling, thus rendering films with improved mechanical properties in comparison to RTD (François & Daraio, 2009).

3.3. ESEM

The improvement of ESEM compared to traditional scanning electron microscopy relates to the possibility of hydrated samples to be examined. The micrograph of the material is acquired in a water vapor environment and this allows the samples to remain intact giving a topography that represents the actual surface structure of the material (James, 2009).

In this work, ESEM micrographs of scleroglucan films obtained without or with FT cycles showed a porous morphology at the edges. Pore sizes of RTD films were bigger than the ones observed for FT films. For instance, the pores in EPS I RTD films exhibited diameters between 1.6 and 3.8 μm, whilst for FT films the pores were in the range of 0.7–1.5 μm (Fig. 2a and b).

A similar trend was observed for all examined *S. rolfsii* ATCC 201126 EPS films. As a general rule, FT-treatment resulted in a more compressed structure indicated by a thicker pore wall, as displayed for EPS I (Fig. 2b). In the same way, previously studied commercial scleroglucan (LSCL) also showed a smaller pore size and a more compact structure when an 8-cycle freeze–thawed sample was examined by ESEM (François & Daraio, 2009).

Accordingly, ESEM observations also pointed to the existence of a reinforced physical crosslinking as a consequence of the FT cycles applied during the preparation of these films. Recently, during the characterization of EPS films from *Pseudomonas oleovorans*, Alves et al. (2011) found that crosslinking reactions probably only took place during the drying process, allowing polymer molecules to get close to each other and pack, as water evaporated. Autocrosslinking reactions were also described for hyaluronic acid hydrogels when using a freezing process (Okamoto & Miyoshi, 2002), and a possible explanation was related to the suppression of repulsive forces between macromolecules thus facilitating their interaction gel formation.

In addition to the scleroglucan film properties herein displayed, it should be stressed the easier dissolution of *S. rolfsii* ATCC 201126 EPSs and the possibility of obtaining clear solutions from these EPSs, as compared to commercial scleroglucans. This fact may be related to an appropriate post-fermentation downstream processing and the high purity grade obtained (Survase et al., 2007; Viñarta et al., 2007).

Considering the global environmental awareness to search for biodegradable packaging films (Alves et al., 2011; Tharanathan, 2003), scleroglucan films may represent a suitable alternative for natural resource conservation, recyclability and the generation of innovative biopackaging. Moreover, as emphasized for other water

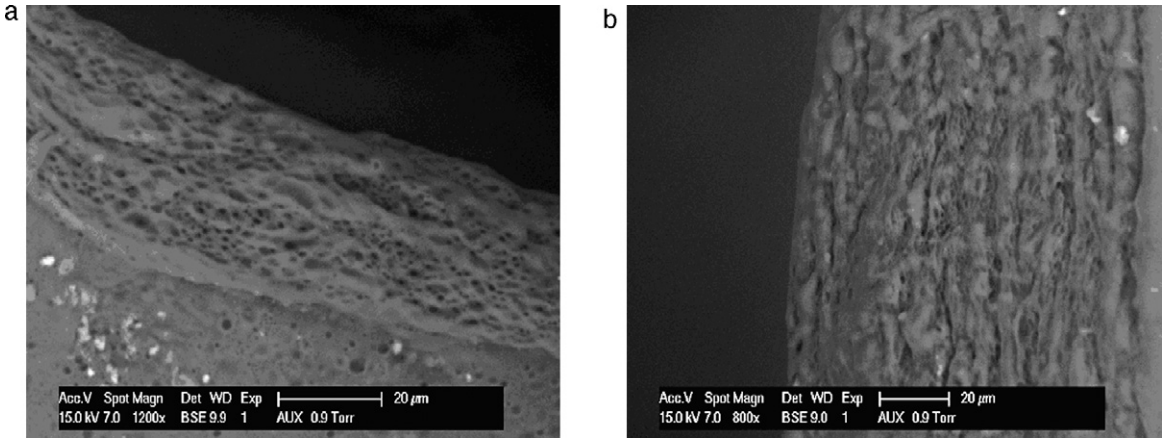

Fig. 2. Environmental scanning electron (ESEM) micrographs of cross sections of EPS I scleroglucan films, observed at the edge. (a) Without freeze–thawing cycles, (b) with 8 freeze–thawing cycles. Scale bars show 20 μm.

soluble microbial polysaccharides (Alves et al., 2011), the possibility to incorporate certain additives to aqueous scleroglucan formulations may expand the use of these films to specific applications.

4. Conclusions

The FT cycling method is suitable for obtaining *S. rolfsii* ATCC 201126 scleroglucan films with a reinforced and more compact network structure due to the existence of a higher number of crosslinking points in these materials, as indicated by swelling, WVT as well as ESEM observations. This process would avoid the use of potentially toxic chemical crosslinkers, allowing the possibility of obtaining biocompatible materials potentially applicable in cosmetic, food and pharmaceutical industries.

This work would also represent a knowledge advance on the *S. rolfsii* ATCC 201126 scleroglucan film characterization considering that polysaccharide features can be somewhat at variance between scleroglucans. Some little variances observed among tested EPSs would reinforce the hypothesis of subtle conformational differences according to the production or downstream processing applied. Nevertheless, it could be said that lab-fermenter scale produced EPSs from *S. rolfsii* ATCC 201126 generally showed a successful behavior for the preparation of reinforced films as compared to commercially available scleroglucan.

The results herein presented open new perspectives for testing these scleroglucans produced at lab fermenter scale for different release systems and biodegradable packaging, thus offering new alternatives to commercial scleroglucan. These achievements may be expected to eventually counteract the monopoly surrounding scleroglucan production on an industrial scale.

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