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Use of microbial levan in edible films based on cassava starch

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ABSTRACT

The objectives of this work were to produce microbial levan using the microorganism *Bacillus subtilis* natto CCT 7712, and to apply this levan in edible films based on cassava starch to obtain new biopolymer blends with functional properties for use in food protection. The films were produced by solution casting, four formulations were produced with different starch:levan proportions (100:0, 90:10, 80:20 and 70:30), and glycerol was used as a plasticizer (20 g/100 g solids). All formulations resulted in films that had a good appearance, without bubbles or cracks. Atomic-force microscopy of the films showed a compact and homogeneous structure, indicative of good compatibility between the polymers. The addition of levan resulted in films with higher solubility, tensile strength and elongation and lower water vapor permeability. Microbial levan was shown to be an interesting functional ingredient for use in edible starch films, improving their barrier and mechanical properties. It can be a cost-effective alternative because it enables the use of levan in mixtures with a low-cost, high-availability material such as starch.

1. Introduction

In the last two decades, increased consumer interest in natural food products has driven research and development of natural active compounds obtained from renewable sources. Several food hydrocolloids have been studied and applied in different functions such as thickeners, gelling agents, stabilizers, and fat replacers or in production of edible films for food protection (Li & Nie, 2016).

Levan is a seldom explored hydrocolloid that could be used more in the food industry. It is a fructose homopolysaccharide in which the fructose units are mainly linked by β(2 → 6)-glycosidic bonds, with some β(2 → 1) linked branch chains, and carries a D-glucosyl residue at the end of the chain. This biopolymer has several interesting properties such as biocompatibility, biodegradability, renewability, flexibility, and eco-friendliness (Shih, Yu, Shieh, & Hsieh, 2005; Srikanth, Reddy, Siddartha, Ramaiah, & Uppuluri, 2015). Dahech et al. (2011) reported that levan has biomedical anti-oxidant, anti-inflammatory, anti-carcinogenic, anti-AIDS and hyperglycemic inhibitor properties. The prebiotic action of levan has also been recognized (Santos-Moriano et al., 2015; Visnapuu, Mardo, & Alamäe, 2015).

Levans are naturally found in many plants and microbial products. A few grasses produce levan as a storage carbohydrate (Moosavi-Nasab, Layegh, Aminlari, & Hashemi, 2010), but it is degraded in growing seasons to provide the plant with the carbohydrates required for grain filling (Gupta et al., 2011). On the other hand, microbial levan is produced from sucrose-based substrates by extracellular levansucrase and has a high molecular weight and extensive branches (Moosavi-Nasab et al., 2010).

The microbial production of levan meets the need for large-scale production, and several microorganisms are reported as levan producers, such as *Erwinia herbicola* (Keith et al., 1991), *Zymomonas mobilis* (Jang et al., 2001; Oliveira, da Silva, Buzato, & Celligoi, 2007), *Microbacterium laevaniformans* (Moosavi-Nasab et al., 2010), *Bacillus subtilis* (Esawy et al., 2011, 2013), *Bacillus subtilis* natto (Dos Santos, Pineda, Celligoi, & Cavalcanti, 2013; Ing-Lung, Tsaur-Chin, Shou-Zoo, & Gen-Der, 2011; Shih et al., 2005) and *Bacillus methylotrophicus* (Zhang et al., 2014).

In this work we used *Bacillus subtilis* natto to produce levan. In previous studies, *Bacillus subtilis* natto was very efficient in producing this biopolymer (Dos Santos et al., 2013). Several applications have been proposed for levan and its use to produce edible films in combination with starch has not been reported. Concurrently, there is an increasing demand for healthy food products from health-conscious consumers, and the development of functional and active packaging systems to preserve food against adverse environmental conditions throughout its shelf life is aligned with these consumers' needs

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(Bersaneti, Mantovan, Magri, Mali, & Celligoi, 2016; Debiagi, Kobayashi, Nakazato, Panagio, & Mali, 2014; Li & Nie, 2016; Navarro, Fratianni, Cozzolino, Granese, & Coppola, 2016).

Additionally, levan can be considered a potential and functional biopolymer to be used in food industry as edible film or coating considering its film forming properties (Srikanth et al., 2015), good oxygen barrier (Öner, Hernández, & Combie, 2016), neutral taste and odor (Öner et al., 2016; Srivastava, Zhurina, & Ullrich, 2009). Levan is a high-cost polymer and the use of low-cost fermentation substrates can reduce its production costs (Oliveira et al., 2007; Öner et al., 2016), however the incorporation of levan in a starch-based film can be considered a feasible alternative to reduce the production costs of the final product.

Thus, the objectives of this work were to produce microbial levan using the microorganism *Bacillus subtilis* natto CCT 7712, and to apply this levan in edible films based on cassava starch to obtain a new biopolymer blend with functional properties for use in food protection.

2. Material and methods

2.1. Materials

Cassava starch was provided by Yoki Alimentos S.A (Paraná, Brazil), and glycerol was purchased from Synth (Labsynth, São Paulo, Brazil). *B. subtilis* natto CCT 7712 (employed for levan production) was isolated from fermented soybeans, a Japanese food called "natto," at the Department of Biochemistry and Biotechnology of the State University of Londrina (Brazil) and identified by Fundação André Tosello (Campinas, Brazil).

2.2. Levan production

Levan was produced in a bioreactor (Bio-tec Tecnal, Piracicaba, Brazil) employing a fermentation medium (2 L) described by Euzenat, Guibert, and Combes (1997), containing 185 g L^{-1} sucrose, 2.0 g L^{-1} yeast extract, 1.0 g L^{-1} KH_2PO_4, 3.0 g L^{-1} $(NH_4)_2SO_4$, 0.6 g L^{-1} $MgSO_4.7H_2O$, 0.2 g L^{-1} $MnSO_4$ and 0.25 g L^{-1} ammonium citrate, with an initial pH of 6.5 for 24 h at 37 °C and 150 rpm. The resulting levan was subjected to precipitation using a proportion of 1.5 parts of absolute ethanol to 1.0 part of fermentation medium. The precipitate was dialyzed for 48 h and lyophilized for use in edible films. Size exclusion chromatography was employed to estimate the molecular mass of the levan using a Sepharose 6B column (Sigma-Aldrich, Missouri, USA) and 50, 150, 410 and 670 kDa dextran standards (Sigma-Aldrich, Missouri, USA). A mixture of levan with two molecular mass ranges was obtained and used for films production, 46.3% ≥670 kDa and 53.7% ≤50 kDa.

2.3. Edible film production

The edible films were prepared by casting with cassava starch, levan and glycerol (plasticizer). For each experiment, the films were prepared with a fixed concentration (3 g/100 g filmogenic solution) of solids (starch + levan) and a fixed glycerol concentration (20 g/100 g solids). Four different film formulations were obtained with different starch:levan proportions (100:0, 90:10, 80:20 and 70:30), and the resulting films were labeled as CS100:L0, CS90:L10, CS80:L20 and CS70:L30, respectively. Cassava starch and glycerol were directly mixed with distilled water and maintained at 95 °C for 10 min with manual shaking. After gelatinization, each starch solution was cooled nearly to 60 °C and mixed with the levan powder, which was easily solubilized. The filmogenic solutions were poured onto circular acrylic plates (0.47 g filmogenic solution/cm^2). The filmogenic solutions were dried (35 °C) in a ventilated oven (model TE-394-3, Tecnal, Piracicaba, SP, Brazil) to a constant weight (approximately 20 h). The resulting translucent films could be easily removed from the plates and were equilibrated at 25 °C and a relative humidity (RH) of 58% for 48 h before testing.

2.4. Film characterization

2.4.1. Thickness

The thickness of the films was determined using a Mitutoyo manual micrometer (São Paulo – Brazil) with an accuracy of ± 1 μm at 10 random positions on the film. The mean standard deviation within the film was approximately 5% of the average thickness.

2.4.2. Atomic-force microscopy (AFM)

AFM was performed using a NanoSurf FlexAFM microscope (Liestal, Switzerland). The analysis was conducted in air, and the images were obtained in intermittent contact mode. The scan was performed in the free oscillation frequency with different amplitudes, depending on the stability and contrast obtained. The set point was 30–50% of the amplitude.

2.4.3. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

The ATR-FTIR spectra of the films were recorded using a Shimadzu FTIR Model IR Prestige-21 (Shimadzu, Kyoto, Japan) equipped with a Pike Miracle ATR. The measurements were performed in triplicate, and 100 scans were conducted in the 4000–700 cm^{-1} spectral range. The spectral resolution was 4 cm^{-1}.

2.4.4. Differential scanning calorimetry (DSC)

DSC analyses were performed using a Shimadzu DSC 60 (Japan) calorimeter. Approximately 3.0 mg of each sample was placed in platinum containers. Each sample was heated from 30 to 225 °C at a heating rate of 5 °C min^{-1} in a helium atmosphere. An empty pan was used as a reference.

2.4.5. X-ray diffraction (XRD)

The crystallinity of each sample was investigated using X-ray diffraction (XRD). The analysis was performed using a PANalytical X´Pert PRO MPD diffractometer (Netherlands) with copper Kα radiation (λ = 1.5418 Å) and operational conditions of 40 kV and 30 mA. All assays were performed with a ramp rate of 1°/min.

2.4.6. Solubility

Film solubility in water was measured as the percentage of dry matter of the film solubilized in water during a period of 24 h (Gontard, Guilbert, & Cuq, 1992). The initial dry matter of each film was obtained after drying the film specimens in desiccators containing anhydrous calcium chloride for a week. The samples were weighed before immersion in 80 mL of distilled water at 25 °C with constant agitation. The film that was not solubilized in water was separated by centrifugation and dried to determine the weight of dry matter. The tests were performed in triplicate.

2.4.7. Water vapor permeability (WVP)

WVP tests were conducted using the ASTM method E96 (2000). Each film sample was sealed over a 0.00181 m^2 circular opening in a permeation cell at 25 °C in a desiccator. To maintain a 75% RH gradient across the film, anhydrous calcium chloride (0% RH) was placed inside the cell, and a saturated sodium chloride solution (75% RH) was placed in the desiccator. The RH inside the cell was always lower than that outside it, and the water vapor transport was determined from the weight gain of the permeation cell. After steady state conditions were reached (approximately 2 h), the cell was weighed every 2 h for 24 h. Changes in the weight of the cell were recorded to the nearest 0.0001 g and plotted as a function of time. The slope of each line was calculated by linear regression ($r^2 > 0.99$), and the water vapor transmission rate (WVTR) was calculated as the slope of the straight line (g/s) divided by the transfer area (m^2). After the permeation tests, the film thickness was measured, and the WVP (g Pa^{-1} s^{-1} m^{-1}) was calculated as WVP = [