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Supercritical assisted enzymatic membranes preparation, for active packaging applications

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ABSTRACT

Materials with antimicrobial activity were obtained by incorporation of an enzyme in cellulose acetate (CA) membranes, to be used for active packaging applications. Membranes were generated by supercritical assisted phase inversion and lysozyme was used as the model enzyme.

Suspensions with different combinations of polymer/enzyme were processed at several operative conditions and were characterized by morphological, chemical and calorimetric analysis.

Results showed that lysozyme was uniformly distributed in the polymeric matrix and process operative parameters influenced membranes morphology and porosity; the morphology of membranes changed from beads-like to fingers-like when CA concentration was increased from 5 to 10% w/w, whereas it was cellular at CA contents of 15 and 20% w/w.

Lysozyme release tests were performed to analyze release kinetics of the investigated membranes. The antimicrobial activity of lysozyme in CA membranes was tested against a suspension of *Micrococcus lysodeikticus*. Activity was divided in two aliquots referring to mobile and immobilized enzyme. Finger-like CA membranes showed the maximum mobile lysozyme activity due to the opened internal structure; the longest activity time measured for cellular membranes was about 90 h. Cellular CA membranes also showed a high immobilized enzyme activity and were able to kill the model microorganism when in direct contact with it.

1. Introduction

Consumers' demand for more natural foods and for environmental protection, catalyzed during the past decades the development of new packaging materials [1], creating functional and active packaging. These materials interact with the product or the headspace between the package and the food system, to obtain the desired outcome [2–4]; antimicrobial food packaging reduces, inhibits or retards the growth of microorganisms that may be present in the food or packaging material itself [5], to extend the shelf life of the packed food.

Traditionally, antimicrobial agents are directly mixed into the initial food formulations. However, direct addition may result in a decrease in the concentration of the antimicrobial agent on the food surface, due to its diffusion into the interior parts of the food. Therefore, the minimum concentration required for the inhibition of the microbial growth may not be achieved and the antimicrobial compound cannot selectively target the food surface [6]. In addition, the neutralization of the added agent, due to interactions with the food components, may occur [5].

For these reasons, the most important desired property of the antimicrobial packaging materials is the controlled release of the antimicrobial agent to the food surface [7–10].

Some authors tried to control the release of the active agent using a three layered film structure. The first layer is the outer barrier layer, whose function is to prevent the migration of the agent to the environment, the second is a matrix layer containing the active agent, and the third controls the release of the active agent to the food [11]. Mastromatteo et al. [12] and Buonocore et al. [13] also developed multilayer films consisting of two external control layers and an inner layer containing the active agent.

Other authors tried to manipulate the release kinetics of the active compounds by changing the degree of cross-linking of the polymer matrix [14].

Gemili et al. [15] produced porous asymmetrical cellulose acetate films for food packaging applications by dry phase inversion, using lysozyme. Indeed, lysozyme possesses enzymatic activity against the beta 1–4 glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine found in peptidoglycan of the bacteria [16]. They tried to control lysozyme release rate changing the degree of asymmetry and porosity of the films. However, overall enzyme release times ranging between about 30 and 300 min were obtained, which are too short to produce a significant increase of the shelf life of a packaged food.

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The techniques proposed in the literature show some limitations: the longest release time of the active agent is short (about one day) even when the multilayer configuration is adopted; a slowdown of the release rate occurs when a multilayer configuration is used. The degree of cross-linking strongly influences both the release kinetic and the amount of active agent released at equilibrium; this fact can be explained considering that part of the preservative loaded into the film is chemically bonded to the polymer backbone via the cross-linking reaction [13].

Supercritical CO_2 (SC-CO_2) based processes have been frequently suggested to overcome the limitations of traditional techniques, namely, to control particle size, [17–21] and organic solvent elimination [22,23]. More specifically, SC-CO_2 assisted phase inversion has been proposed to produce membranes of some polymers with controlled morphology and porosity [24–27]. Membranes and aerogels loaded with pharmaceutical [28–31], catalyst [32,33] and active agents for biomedical applications [34,35] have also been proposed using this technique.

More specifically, cellulose acetate (CA) membranes have been prepared from polymer-acetone solutions at various polymer concentrations (from 5 to 40% w/w in CA), temperatures (between 45 and 65 °C) and pressures (from 100 to 200 bar) obtaining cellular or beads-like structure. The control of CA membranes' morphology has been achieved [24].

However, to the best of our knowledge, SC-CO_2 assisted phase inversion has never been used to produce enzyme or biologically active compound loaded membranes.

To overcome the limitations previously discussed in the case of enzyme loaded polymers and, specifically, their too short release time, in the present work the production of cellulose acetate membranes by SC-CO_2 assisted phase inversion, loaded with lysozyme, is proposed, to obtain an internal packaging device [13,15,36].

As previously discussed, this technique allows to control the morphology and porosity of the membranes obtained, and, therefore, can also allow the control of lysozyme release times. Enzyme unfolding can be preserved during processing *maintaining small water quantities in the membrane*. The processing times are short and negligible organic solvent residues are present in the produced membranes.

Lysozyme loaded membranes will be characterized in terms of porosity, enzyme release and activity against *Micrococcus lysodeikticus*, used as a model microorganism.

2. Experimental section

2.1. Materials

Cellulose acetate, CA, (average Mn ca. 50,000 with acetyl content of 39.7%), acetone (purity 99.5%), lysozyme from chicken egg white and *M. lysodeikticus* (Sigma M3770) were bought from Sigma-Aldrich (Milan, Italy); CO_2 (purity 99%) was purchased from S.O.N. (Società Ossigeno Napoli, Italy); distilled water was produced in our laboratory using ISCO mod. AUTOSTILL DST/5. All materials were processed as received.

2.2. Enzymatic membranes preparation

Polymer solutions were prepared by solubilizing cellulose acetate in acetone; the solution containing lysozyme and water was separately prepared and, then, poured in the polymeric solution and mixed for 40 min. Solutions with CA contents of 5, 10, 15 and 20% w/w, and a fixed quantity of 1.5% w/w lysozyme and 5% w/w water were prepared. The solution was distributed on stainless steel caps, with a diameter of 2 cm and a height of about 800 μm, and, then, processed.

Membranes were produced in a home-made laboratory apparatus previously described [37]. When operative temperature was reached, the caps were rapidly put inside the membrane preparation vessel (a 316 stainless steel vessel with an internal volume of 80 mL) to minimize the evaporation of the solvent. The vessel was closed and filled from the bottom with SC-CO_2 up to the desired pressure, using a high pressure pump (Milton Roy–Milroyal B, Pont-Saint-Pierre, France) in about 10 min. Then, the vessel was flushed with CO_2 for 3 h, and, depressurized in about 30 min, collecting the dried membranes.

2.3. Enzymatic membranes characterization

2.3.1. Scanning electron microscopy (SEM)

Enzymatic membranes were cryofractured using liquid nitrogen (SOL, Milan, Italy); then, the samples were sputter coated with gold (AGAR Auto Sputter Coater mod. 108A) at 30 mA for 150 s and were analyzed by a scanning electron microscope (SEM) (mod. LEO 420, Assing, Italy), used to study the membrane structure and pore size.

2.3.2. Membrane pore size analysis

Sigma Scan Pro 5.0 (Jandel Scientific, San Rafael, CANADA) and Origin 8.5 (Microcal, Northampton, USA) softwares were used to determine the average diameter of membrane pores. Images taken at various locations in the membrane were used for each calculation. We measured about 300 pores for each sample analyzed. Using Origin software, we first represented a histogram with the percentage of the pores having a given diameter, then we performed a curve fitting to obtain the distribution curve.

2.3.3. Membrane porosity

The porosity (ε) represents the 'void space' of the membrane and was calculated from the density of the membrane (ρ_S = Membrane volume/Membrane weight) and the density of untreated CA

$$\varepsilon = 1 - \frac{\rho_S}{\rho_P}$$

The membrane density was determined by measuring its volume and weight. The membrane volume was obtained using Archimede's principle: the membrane was waterproofed and subsequently immersed in pure water. Calculating the weight of the displaced water, we measured the volume of the sample. The presence of lysozyme was neglected in this calculation.

2.3.4. Energy dispersive X-ray spectroscopy (EDX)

Enzymatic membrane samples were cryofractured using liquid nitrogen and sputter coated with chrome (EMITECH K575X peltier cooled);, then they were analyzed by energy dispersive X-ray spectroscopy (EDX) (INCA Energy 350, Oxford Instruments) to identify the elements that constitute the samples, in particular, sulfur atoms that can be representative of lysozyme dispersion within the polymeric matrix.

2.3.5. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) (DSC 30 Mettler Toledo) was carried out to analyze and identify any changes in the thermograms of pure substances compared to polymer/enzyme formulations.

Calorimetric analysis was performed in the temperature range between −60 and 300 °C, with a heating rate of 10 °C/min; the inerting gas was nitrogen, with a flow rate of 50 L/min.