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Development of an antifungal film by polyethylene surface modification with natamycin

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ABSTRACT

An antifungal low-cost film for cheese packaging was developed using a tailor-made coating process by direct natamycin (Nat) spraying onto heat softened polyethylene film surface. Two spraying systems Nat/ethanol solution and Nat/n-heptane suspension with the same Nat concentration were explored. To select the adequate system, differences in coating efficiency regarding shape, size, dispersion and distribution of particles, as well as polyethylene film/Nat adhesion and antifungal activity of both modified films were assessed and compared. Besides, the suitability of final product related to its use as food flexible packaging was considered. The results indicate that Nat coating is achieved either by direct particle incorporation (from a suspension) or by its *in-situ* formation (from a solution). Both kind of Nat coated films have shown antifungal effectiveness against *A. niger* even after severe extraction treatments. The proposed versatile process is feasible to be industrially scaled and allows to obtain an antifungal film suitable for cheese packaging that can be intensively handled without losing their activity.

1. Introduction

Current trend in food packaging technology is focused on their active participation to extend food shelf-life, maintaining and even enhancing its quality and safety (Yildirim et al., 2018). Active packaging achieves this purpose through a dynamic interaction between package, food and their micro-environment. In this context, as microbial contamination is one of the main reasons of food spoilage (Rawat, 2015; Subramaniam & Wareing, 2016), the development of active packaging with antimicrobial properties is crucial. With particular emphasis on extending food shelf-life and avoiding losses, food antimicrobial packaging works reducing, inhibiting or retarding the microorganism growth in both, food and packaging material (Appendini & Hotchkiss, 2002; Malhotra, Keshwani, & Kharkwal, 2015; Van Long, Joly, & Dantigny, 2016; Yildirim et al., 2018).

Dairy products are perishable food very susceptible to microbiological spoilage, particularly to contamination by yeasts and molds. Fungal spoilage renders cheese unfit for human consumption, resulting in huge economic losses for the dairy industry (Fernandez et al., 2017). Benkerroum (2016) claims that cheese industry is one of the most affected by mycotoxins because of fungal spoilage. Therefore, antifungal agents are either applied directly on each product during processing or included into the package. In the first case, cheese taste could change, so external use of antifungal agents is more recommended. In this way, it is usually added to cheese surface by spraying, dipping or coating. However, these methods have low efficiency because of both active agent degradation and its possible diffusion in cheese (Basilico, Debasilico, Chiericatti, & Vinderola, 2001; Reps, Drychowski, Tomasik, & Winiewska, 2002). On the other hand, the use of antifungal agents for fresh cheese preservation is not usual, mainly due to its short life. However, supermarkets suffer big losses of these kind of cheese because its high water activity and moisture content allow for microbial spoilage (Evert-Arriagada, Hernández-Herrero, Juan, Guamis, & Trujillo, 2012; Fernandez et al., 2017; Garnier et al., 2017; Ho, Howes, & Bhandari, 2016; Hwang & Gunasekaran, 2001; Sedaghat, Eskandari, Moosavi-Nasab, & Shekarforoush, 2016). Consequently, to develop an easy handling low-cost antifungal package seems to satisfy this particular big market niche. The use of antifungal active packaging whose concentration of active agent remains constant for relative long time near the food surface seems to be a possible solution for fresh cheese package. Furthermore, including antifungal active agents on the packaging has the further advantage of avoiding the need of cheese special coating. Thus, active packaging provides a two-in-one solution as it includes the packaging and the antifungal activity. In fact, since packaging is always needed, antifungal active packaging could introduce both the advantages of an increased fresh cheese shelf life and

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diminish overall costs (Bastarrachea, Wong, Roman, Lin, & Goddard, 2015; Haghighi-Manesh & Azizi, 2017).

Regarding the antifungal agent, the increasing trend is using compounds isolated from different natural sources instead of chemically synthetized compounds because of consumer awareness concerning foods preserved with natural additives (Del Nobile, Lucera, Costa, & Conte, 2012; Ribes, Fuentes, Talens, & Barat, 2017; Yildirim et al., 2018). Natamycin is a natural antifungal agent widely used in dairy industry because of its good cost/efficiency relationship (Atta, Selim, & Zayed, 2012; Brik, 1994; Davidson, Sofos, & Branen, 2005; Jalilzadeh, Tunçtürk, & Hesari, 2015). It has been approved as a food additive in over 40 countries and has been considered as a GRAS product by the FDA and can be safely used for surface cheese treatment according to EFSA (Resa, Jagus, & Gerschenson, 2014; EFSA, 2009). For these reasons, various alternatives of cheese active packaging incorporating natamycin into different polymer matrices have been studied (Ture, Eroglu, Ozen, & Soyer, 2011; Fajardo et al., 2010; de Oliveira, de Fátima, Soares, Pereira, & de Freitas Fraga, 2007; Var, Erginkaya, Güven, & Kabak, 2006; Reps et al., 2002; Hanušová et al., 2010; Basilico et al., 2001).

On the other hand, polyethylene (PE) is the most used synthetic polymer for food flexible packaging because of its good cost/performance/processability relationship (Prasad & Kochhar, 2014; Robertson, 2016). However, it is strongly inactive itself having an inert surface and needs to be modified in order to acquire antifungal activity. In literature, there are some works focused on the development of antifungal PE packaging using natamycin as active agent. Shin, Liu, Chikthimmah, and Lee, (2016) immobilized Nat in a Low-Density PE (LDPE) by binding it through acrylic acids grafted to PE surface using ultraviolet treatment. Hanušová et al. (2010) have coated PE films using polyvinyldichloride lacquer loaded with natamycin. Cong, Zhang, and Dong, (2007) have evaluated an antifungal coating based on PE wax microemulsion containing Nat. These methodologies seem to be efficient for fungal prevention, but they involve not easy processing or generate non-recyclable materials.

In order to obtain active packaging, material surface modifications with active agents seems to be more convenient than their incorporation inside the film matrix. In the first case, all active agents will be in contact with the product, minimizing the amount required to impart efficacy and therefore lowering costs, whereas in the second case, the active agents must diffuse inside the film and then act over the food (Bastarrachea et al., 2015). The potential success of selected surface modification methods is defined by its competitive advantages, mainly the economic ones, but also the adapting versatility to the specific market demands including “taylor-made” solutions and the global sustainability of their implementation. In this case, those methods which allow the incorporated active agent quantification, are easily adaptable to continuous production systems, consume little energy and do not hinder their post-consumer recycling are preferable. In turn, the obtained active material must be suitable for food use and must not lose its active power after suffering packaging process handling and the attrition caused by direct contact with the packaged food.

In this work, a low-cost antifungal film for flexible packaging system is developed. Its main use is for cheese packaging, mainly focusing on fresh ones. The proposed system is based on direct incorporation of natamycin (Nat, food commercial additive) onto PE film surface. This development resulting in Nat coated film could be used directly in both cheese factory and retailing to increase cheese shelf life and food safety. This surface modification involves an easy to use methodology during film production; i.e. in the calendaring step of continuous cast film extrusion and avoiding adhesives use. A tailor-made coating process by direct Nat spraying onto softened PE film surface using less than 1% w/w of Nat is proposed. Two different spraying fluids are explored: Nat/ethanol solution and Nat/n-heptane suspension with the same Nat concentration. Coating efficiency of each system was evaluated regarding shape, size, dispersion and distribution of particles as well as PE film/Nat adhesion and antifungal activity.

2. Materials and methods

2.1. Materials

Blown films of low density PE from Dow-Polisur, LDPE 203 (Mw: 229,300 g/mol, Mn: 22,500 g/mol), 80 μm thick, were used. Typical commercial natamycin containing 50% pure natamycin and 50% Lactose (weight percentages) was used as active agent. Two different solvents: N-heptane (≥99.5% purity and Absolut Ethanol (≥99.5% purity), both from Cicarelli were used.

2.2. Surface modification method

PE film squares of 225 cm^2 (15 × 15 cm) were softened by heating only in its surface and then, sprayed with 20 ml of Nat/ethanol solution or Nat/n-heptane suspension, both with the same concentration of Nat: 1 mg/ml and at room temperature, assuring that 20 mg of Nat remains onto PE film surface. Used heating system is composed by 2 conventional infrared lamps and an aluminum reflector with adjustable vertical distance from the front side film substrate. The surface film temperature was sensed and controlled to be 95 ± 5 °C with a thermocouple during the spraying procedure. This temperature assures that the polymer is softened but not melted (Grafia, Martini, & Barbosa, 2018). The spray equipment used presented a conventional 120° fan nozzle connected to a dry air stream at 3 bars, room temperature, at a flow rate of 6 ml/minute, placed 15 cm away from the film surface supported in aluminum plates. Modified films using ethanol (PENatE) or n-heptane (PENatH) could be used immediately afterwards the modification procedure as solvents are evaporated during spraying.

2.3. Extraction/Adhesion test

In order to evaluate particle adhesion to film surface and consequently coated film response to manipulations of cheese flexible containers, severe tape extraction and water sonication experiments were carried out. In this way, coated films were held firmly in a jig and then a pressure-sensitive tape was attached to the film surface and then, removed pulling it off ; at an angle close to 180° analogous to those described in ASTM D 3359-02-2002. The films treated with this extraction method are identified with the subscript "tape" ($PENatE_{tape}$ and $PENatH_{tape}$). Regarding water sonication, coated films were immersed in water and sonicated for 2 h at room temperature. The last method produces mechanical effect on all particles and lactose solubilisation (Brittain et al., 1991; Machado, Coutinho, & Macedo, 2000; Peña, Daali, Barra, & Bustamante, 2000). Then, after water extraction the remaining particles would be from natamycin. The films treated with this extraction method are identified with the subscript "ws" ($PENatE_{ws}$ and $PENatH_{ws}$).

2.4. Characterization

Nat particles and PE film surface, before and after modification, as well as before and after the adhesion tests, were examined by scanning electron microscopy (SEM) in a Leo EVO-40XVP microscope. Film samples and particles dispersed over aluminum conductive tape were previously coated with Au in a sputter coater PELCO 91,000.

Particle size distribution was calculated by processing SEM images by using Fiji software previously calibrated for the used magnifications. More than 100 particles of each sample were analyzed and the largest length of each particle was selected as the size descriptor. Their mean is reported as size value and the polydispersity of the particle was expressed by the span. Span index is calculated as:

$$Span = (D_{90} - D_{10})/D_{50}$$

where D_{90}, D_{50} and D_{10} are the particle largest length for 90%, 50% and 10% of the sample, respectively.

Chemical evidence of Nat presence over films was assessed by Fourier Transform Infrared Spectroscopy (FTIR) in Attenuated Total Reflection (ATR) mode. Used equipment was a Micro-ATR-FTIR Thermo Nicolet Continuum operated with a resolution of 4 cm^{-1}, 500 scans and in a range of 650-4000 cm^{-1}.

The modification process effect on natamycin crystalline structure were analyzed by X-ray Diffraction (XRD). A Philips PW1710 X-ray diffractometer (Netherlands) was used, which is provided with a tube, a copper anode, and a detector operating at 45 kV and 30 mA with 2θ ranging from 3 to 60°.

Antifungal activity of Nat-coated films, was evaluated by a comparative bioassay employing *Aspergillus niger ANIG 001,* isolated from onions and deposited in the collection of the CERZOS – CONICET was used in this work. These strains were cultivated in 2% Malt Agar (MA) (Britannia) at 25 °C for 15 days in darkness. Identity of fungi was confirmed by optical microscopy. Conidial concentration was determined by counting in a Neubauer Chamber, with the help of an optical microscope under a magnification of 400 × .The conidial concentration was adjusted to 6×10^6 conidia/ml. Fungal biomass was harvested with a Digralsky loop using 15 ml of sterile distilled water for each Petri plate. Using the pour–plated method, 1 ml of this solution was inoculated per plate. Discs of all films were horizontally put in agar (with modified surface in contact to it) as same as Nat particles and incubated at 25 °C, in darkness. After 7 days, they were examined to evaluate fungal growth, sporulation and appearance of inhibition zone. All the assays were done by triplicate.

3. Results and discussion

3.1. Coating efficiency

Coating efficiency as a function of used solvent was evaluated on three aspects: natamycin presence without structure changes, coating coverage and particles adherence onto PE surface. In turn, the coated films from Nat/ethanol solutions and from Nat/n-heptane suspensions were compared and the coating formation mechanism was discussed. Chemical analysis by FTIR was used to determine Nat presence and to avoid chemical structure changes. Dispersion, distribution and adhesion of particles were assessed by a morphological analysis using SEM. The natamycin crystals presence and integrity were corroborated and the modification process effect was analysed by crystallographic studies (DRX).

The first evidence of Nat presence on PE surface was obtained by FTIR. In Fig. 1, FTIR spectra (from 2000 to 900 cm^{-1}) of PENatE, PENatH, PE and Nat are compared. Typical PE and Nat absorption bands are detected on both PENatE and PENatH spectra. Identification and assignment of main absorption bands attributed to natamycin and PE are listed in Table 1. The presence of natamycin absorption bands on the spectra of coated films has demonstrated not only natamycin presence, but also preservation of its chemical integrity, without showing any degradation evidence. On the other hand, PENatE and PENatH spectra present small differences in the relative intensity of some bands. Initially, this observation suggests an influence by the solvent used in the final Nat coated structure, awarded to the difference in its solubility.

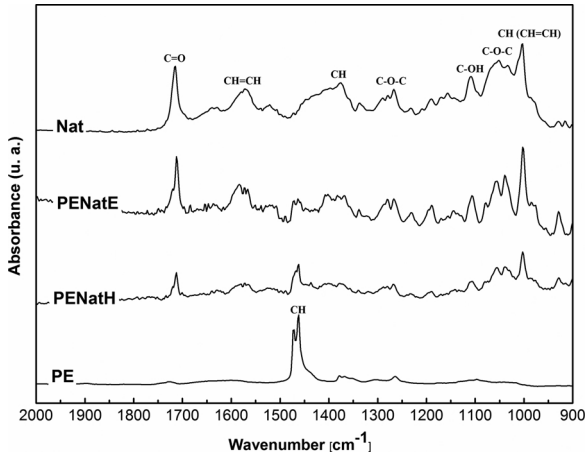

Fig. 1. FTIR surface spectra of PE film, coated films (PENatH and PENatE) and initial natamycin particles (Nat).

Nat particles dispersion and distribution onto PE film surface was assessed by morphological analysis of modified film surfaces. Solvent influence on the same coating aspects was also analyzed. SEM micrographs of PE, PENatH, PENatE and Nat particles at 100x and 5000x are shown in Fig. 2. PE surface exhibits a smooth appearance at both magnifications. On coated films, very similar particles to Nat are observed well dispersed in the whole surface. However, comparing the coating onto PENatH and PENatE, some differences in the amount, distribution and size of Nat particles are evidenced. PENatE seems to have greater coverage than PENatH with higher content of particles. Particles onto PENatH present similar appearance to original Nat. On the contrary, particles onto PENatE seems to be particles aggregates with several shapes.

Results of particle size distribution measurements are shown in Fig. 3. All samples have similar polydispersity, but particles from coated films present a lower mean size than original Nat. This fact agrees with the spraying method used to carry out the modification obtaining greater surface coverage with equal Nat content (20 mg/225 cm^2). Mean size and span index of particles depends on the used solvent. PENatH particles are smaller (in mean) and more homogeneous in size than PENatE. These results can be explained taken into account the different coating mechanism involved in each case. Whereas Nat particles in PENatE surface are originated from a recrystallization, the particles in PENatH are the initial ones dispersed in n-heptane and exposed to attrition during the spraying process. Thus, in the first case, as Nat crystallize in situ from a solution, the aspect of attached particles is different to those in original Nat. On the contrary, in the second case, particles conserve initial appearance but diminish their size because the attrition during spraying process from a suspension.

Fig. 4 shows no appreciable variation in PE crystallinity as expected since PE film was modified only on its surface. This parameter is important because it allows us to infer no changes in both barrier and mechanical properties. In all spectra, typical peaks of PE orthorhombic structure are not modified. The X-ray diffraction scans of PE, PENatE and PENatH (Fig. 4) show a sharp and a small peak in the region of Bragg angle (2θ) between 20° and 25°, corresponding to the plans (1 1 0) and (2 0 0), with similar peaks ratio (Hermans & Weidinger, 1961; Kakudo & Ullman, 1960; Krimm & Tobolsky, 1951; Rizzo, Baione, Guerra, Martinotto, & Albizzati, 2001). These results corroborate the hypothesis of PE film modification confinement to a thin surface layer that does not significantly alter its bulk properties. Our assumption is based on the method used to achieve the PE film natamycin coating by direct adhesion of Nat particles onto a softened PE film. Please note that, short chains melt first than long ones and, according to Helfand and Tagami (1972), the major amount of PE short molecules are localized in film surface. Therefore, the PE film surface is melted at lower temperature than its core. In this way, by an accurate selection of processing window temperature (Grafia et al., 2018), the film core is not affected preserving its crystalline structure.

Fig. 4 also gives information about the effect of film modification processes on Nat crystallinity. Polymorphic forms of some polyene antifungal like natamycin are known (Kwon et al., 2006; Threlfall, 1995; Ghielmetti, Bruzzese, Bianchi, & Recusani, 1976). In fact, it has been found that antifungal polyenes activity towards yeast and fungi can be markedly enhanced by modifying the polymorphic form and/or

Table 1
Identification and assignment of main FTIR absorption bands in PENatE, PENatH, PE and Nat.

Frequency[a] [cm^{-1}]			Assignment		References
PENatE and PENatH	PE	Natamycin			
3500		3500	OH stretching		Brik (1994),
3270		3270	NH_3^+ stretching N—H		Atta et al. (2012)
2942		2942	CH_2 stretching		
3600–2400		3600–2400	—OH of water		
2915	2915		CH_2 asymmetric stretching		Gulmine, Janissek, Heise, and Akcelrud (2002)
2847	2847		CH_2 symmetric stretching		
1712		1715	C═O lactone, conjugated ester		Brik (1994),
1572		1572	CH═CH; COO^-		Atta et al. (2012)
1472 1462	1472 1462		CH bending deformation		Gulmine et al. (2002)
1380		1380	CH stretching		Brik (1994), Atta et al. (2012)
1378	1378		CH_3 symmetric deformation		Gulmine et al. (2002)
1306	1306		CH_3 twisting deformation		
1270		1270	Stretching vibrations C-O	C—O—C de epoxi	Brik (1994),
1110		1110		C—OH asymmetric stretching	Atta et al. (2012)
1056		1056		C—O—C	
1078-1040			Lactose (1075 cm^{-1}-1040 cm^{-1})		Solís-Oba et al. (2011)
1005		1005	CH deformation in CH═CH		Brik (1994), Atta et al. (2012)
721-719	721-719		CH_2 rocking deformation		Gulmine et al. (2002)

[a] Bands assigned to the grafting product are highlighted in bold.

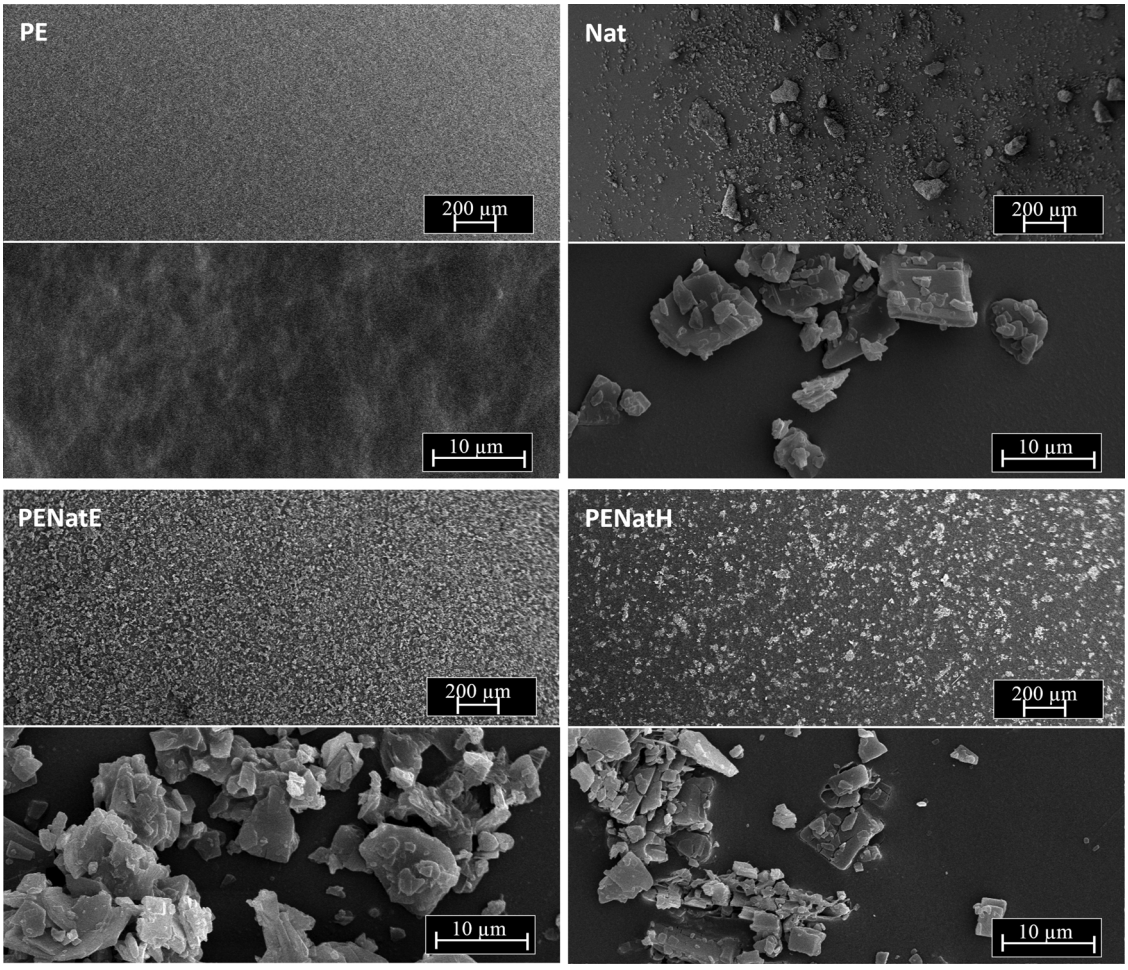

Fig. 2. SEM micrographs (100x and 5000x) of the surface of PE film, Nat particles, and coated films.

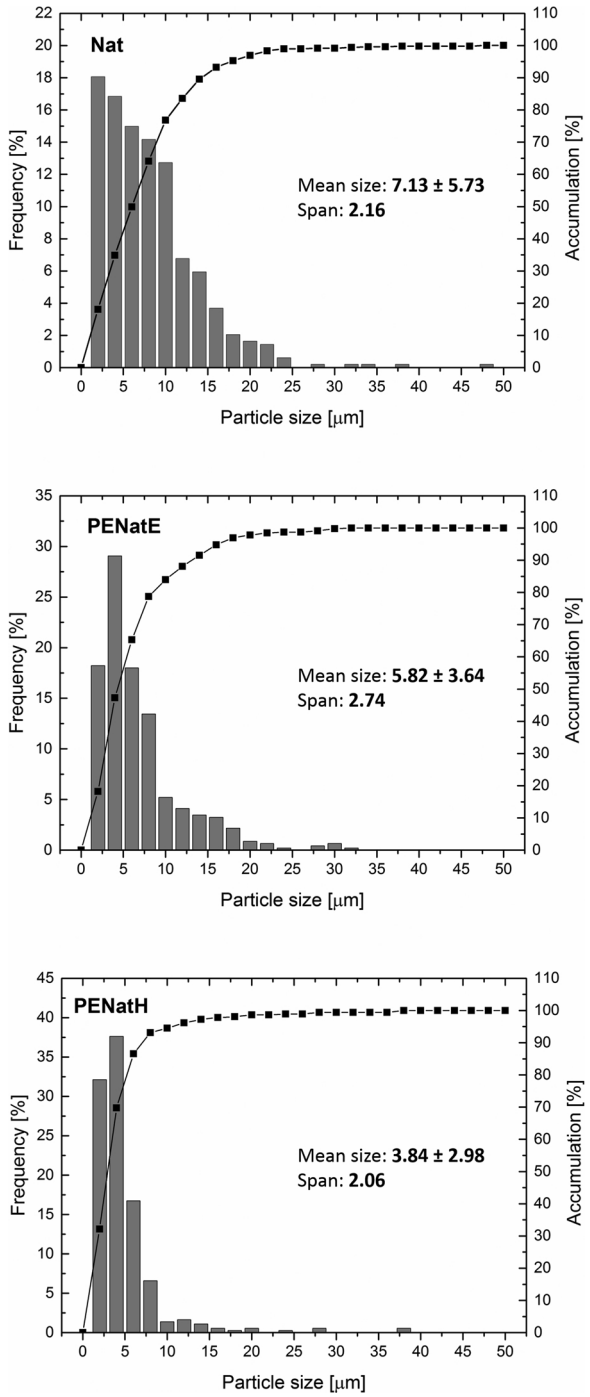

Fig. 3. Particle size distribution histogram and accumulative curve of: Nat, PENatE and PENatH.

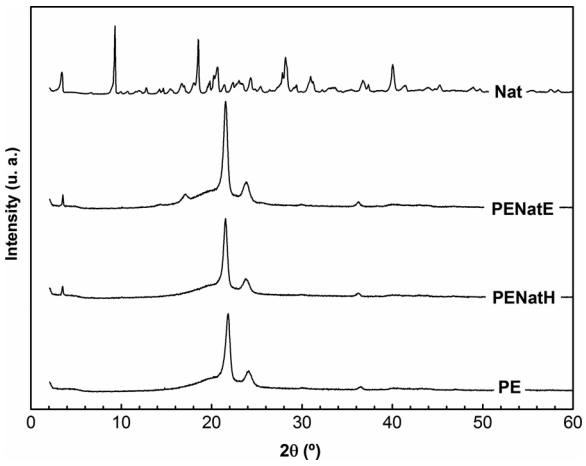

Fig. 4. XRD spectra of Nat particles, PE and coated films (PENatH and PENatE).

dissolved in a suitable solvent system like ethanol (De Haan, Stark, & Bozzetti, 1999). PENatE and PENatH spectra conserve the same peaks of PE spectrum. However, Nat peaks do no match completely with the new peaks in these spectra. A peak around 3.5° attributed to natamycin is detected in the spectra of both coated films although it is not the most intense in original Nat. A new peak at around 17° is detected only in PENatE diffractogram. These results could be explained considering the natamycin polymorphism. At least α-natamycin, γ-natamycin, δ-natamycin and natamycin methanol-solvate have been identify by De Haan et al. (1999). Previously, Brik (1994) mentioned the existence of natamycin in polymorph states that would correspond to this x-ray spectra. The new peak at 17° in PENatE is explained taking into account the natamycin solubility in ethanol and the recrystallization occurring during the coating process. Then, during Nat solution spraying, a high crystals amount with their own crystalline morphology are generated. This claim is also based on the insolubility of lactose either in ethanol or in n-heptane (Brik, 1994; Brittain et al., 1991; Machado et al., 2000; Peña et al., 2000). The other changes observed in both spectra, PENatE and PENatH could be related to ethanol or n-heptane solvation of natamycin, or a heating shock effect when the spray solution/suspension impacts on the softened PE film surface. These changes are not detrimental to the antifungal activity since its preservation was verified.

In order to study particles-PE interactions and their final adhesion, a comparative analysis between coated and coated-extracted films was performed using SEM. Fig. 5 includes SEM micrographs of PE modified films after extraction with tape (PENatE$_{tape}$ and PENatH$_{tape}$) and sonicated under water (PENatE$_{ws}$ and PENatH$_{ws}$) at two magnifications (100x and 5000x). Comparing these micrographs with those of initial coated films (Fig. 2), particle adhesion seems to be independent of the used solvent. Although the particles amount respect to initial coated film diminishes, several particles remain attached to the film surface. Both, initial and extracted coated film using ethanol/Nat solution present a bigger amount of observed retained particles than those prepared with n-heptane/Nat suspension. The stronger the extraction treatment severity, the smaller the number of remaining particles, i.e., PENatE$_{tape}$ and PENatH$_{tape}$ present more particles than PENatE$_{ws}$ and PENatH$_{ws}$. It is important to note that whereas modified films extracted with tapes only present a decrease in the amount of surface particles, those extracted with water and sonication also show holes. On the other hand, while PENatE$_{ws}$ contains particles and holes, PENatH$_{ws}$ seems to contain only holes. Shape and distribution of these holes indicate that they were previously occupied by particles and pulled out. This result reveals different intensity levels in PE/Nat adhesion strength which demonstrates that some particles have penetrated onto PE film remained wholly or partially immersed in it.

The actual solvent influence on coated films surface morphology was assessed analysing aspects like amount, shape, size, and interaction mode of Nat particles with PE. The main differences are evident in samples extracted by sonication under water. Particles on PENatE$_{ws}$ surface maintain the blocky shape with neat edges, but appear cracked, agreeing with disintegration start because of sonication effect, while in PENatH$_{ws}$, it seems that only small particles remain in the holes edges. In addition, the holds also differ: those observed in PENatH$_{ws}$ are bigger and apparently deeper than PENatE$_{ws}$, with structures like "filaments" or "threads" around them as shown in Fig. 6. These structures are consistent with the inclusion of Nat particles in softened PE since the molten polymer chains tend to keep particles attached to the film.

Nat particles are directly incorporated (from suspension) or

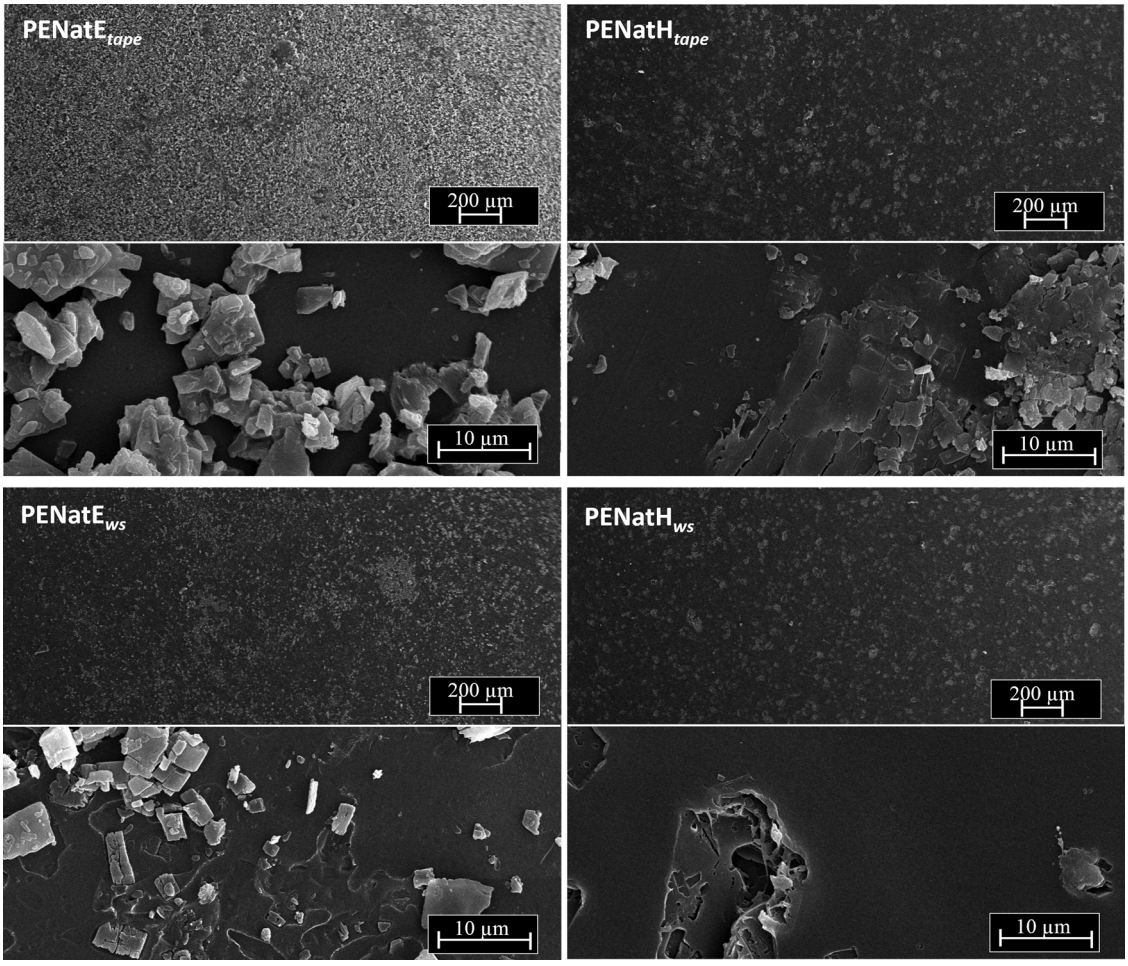

Fig. 5. SEM micrographs (100x and 5000x) of the coated films surface after extraction with tape (PENatE$_{tape}$ and PENatH$_{tape}$) and sonication in water (PENatE$_{ws}$ and PENatH$_{ws}$).

crystallized *in-situ* (from solution) onto whole PE film surface by spraying. The Nat particle inclusion mechanism in PE film surface from a Nat-ethanol solution involves an in-situ natamycin crystallization with insoluble lactose acting as nucleation points (Brik, 1994; De Haan et al., 1999). This in-situ generated crystals have their own crystalline structure not necessary equal to the original one as was corroborated by XRD (Fig. 4). During the spraying process, ethanol is evaporated, lactose particles impact on the surface of the polymer in semi-molten estate and natamycin crystal grow up from it. When the polymer is cooled and solidifies, natamycin particles remain trapped with one part inside and other outside the polymer, i.e. semi-included in film surface.

When spraying from a suspension (Nat/n-heptane), Nat particles, finely divided by the spraying, form a layer on the film surface remaining adhered by interaction with the semi-molten polymer. The Nat incorporation into the softened film surface involves sedimentation of the particles in the polymer by their own weight plus the force of

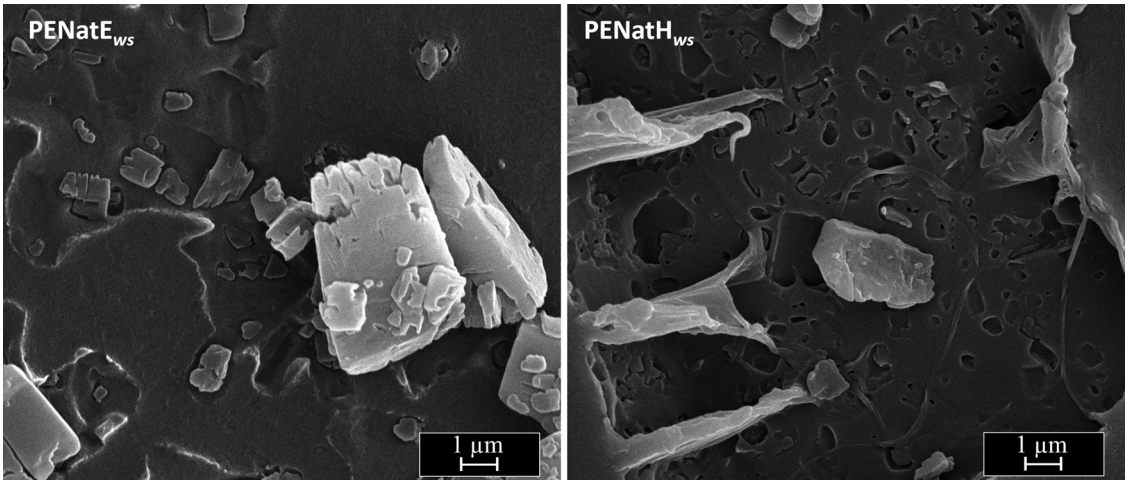

Fig. 6. SEM micrographs (15000x) of the both coated film surface after sonication in water (PENatE$_{ws}$ and PENatH$_{ws}$).

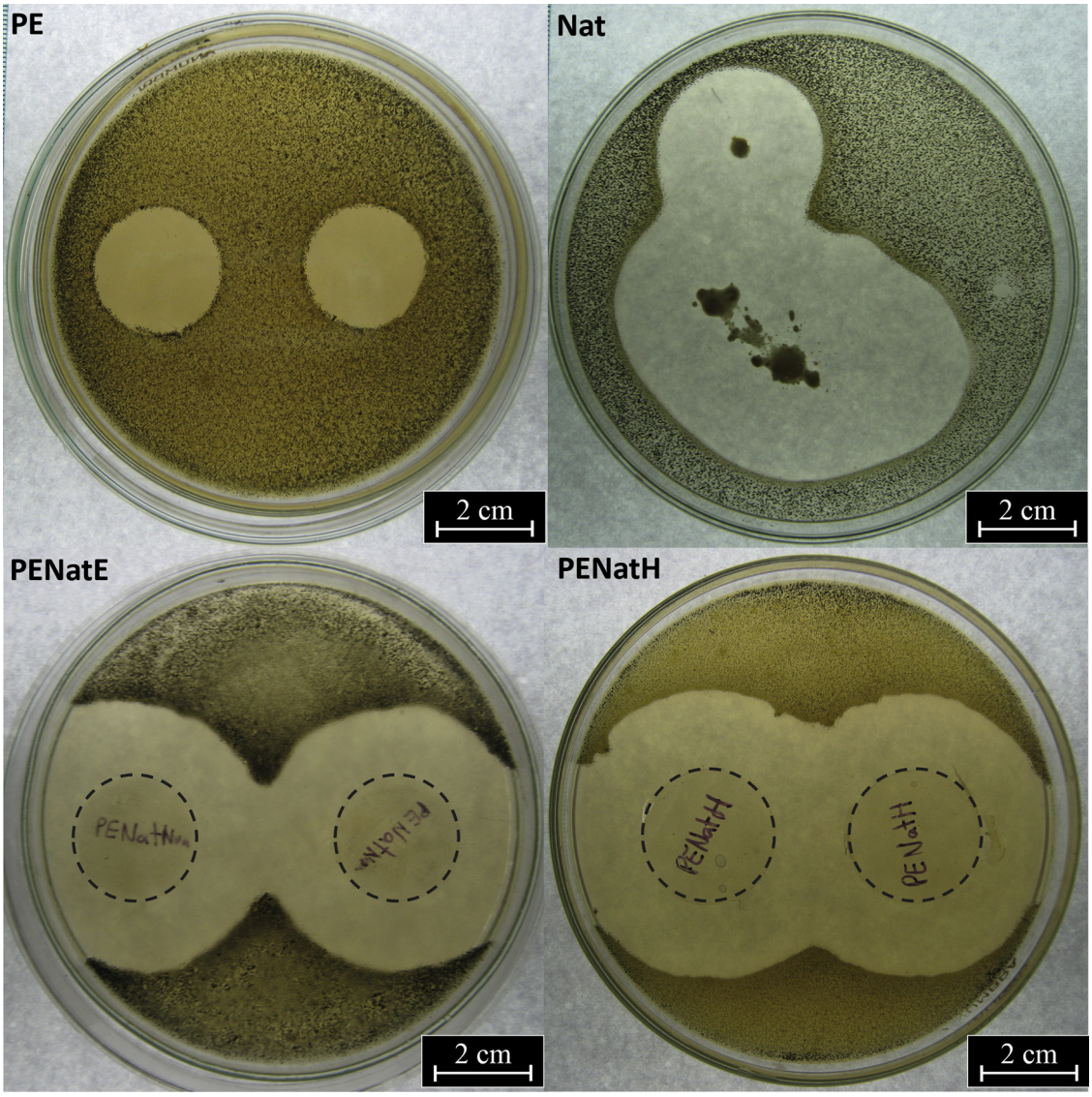

Fig. 7. Inhibition zone of PE film (control), Nat particles (control) and coated films (PENatE and PENatH) against *A. niger*.

atomization against the viscous impediment exerted by the molten polymer. Particles partially penetrate the molten film surface and are retained as the polymer solidifies.

In addition, and to comprehend particles final morphology onto PE films it is important to consider that chemical affinities between each pair Nat-solvent and solvent-PE are opposite. While ethanol has greater affinity for natamycin than for PE, n-heptane has greater affinity for PE than for natamycin. Also, both solvents can swell PE, but ethanol has a lower swell rate than n-heptane (Lützow et al., 1999; Asfour, Saleem, & De Kee, 1989; Pinsky, 1957; Hilton & Nee, 1978). Hence, they affect differently not only natamycin crystals, but also their penetration in PE (Brik, 1994; Van Krevelen, 1997).

Clearly, the proposed method for natamycin attaching onto PE surface allows us to obtain a film covered with antifungal particles spraying them both in suspension and in solution. In both cases, some particles remain attached directly to PE, while others are attached only by static electricity or friction. However, as discussed above, attached Nat suffer crystallinity changes. Then, to assess if this fact, together with the severe process conditions used for film modification produce changes in natamycin antifungal activity, all films were tested, and the results are discussed in the next section.

3.2. Antifungal activity

Antifungal activity of Nat-coated PE films was assessed against *A. niger* strains. This kind of fungi is a problematic food contaminant that produces a variety of secondary metabolites, some of them with toxic effects (Gravesen, Frisvad, & Samson, 1994). The activity of natamycin against *A. niger* is well documented (Atta et al., 2012, Pedersen, 1992). However, this activity could be affected during coating process because of the heat treatment, or changes in natamycin crystalline structure when ethanol is employed as solvent (Brik, 1994; De Haan et al., 1999). It was demonstrated that stability and activity of natamycin are not affected when it is exposed at temperatures up to 120 °C for one hour (Brik, 1994). In the proposed modification process, Nat is spraying at circa 95 °C for a maximum of 5 min. Then, it is possible to infer that natamycin remains active after the PE coating process. On the other hand, it was reported that natamycin crystallized from ethanol enhances their antifungal activity (De Haan et al., 1999).

Antifungal activity was determined using Kirby-Bauer disk diffusion test (Hudzicki, 2009) by measuring the inhibition zones formed around films and active agents. Fig. 7 shows disk diffusion test of film control (PE), Nat particles and coated films (PENatE and PENatH) against *A. niger*. Natamycin strongly inhibited growth of *A. niger* unlike PE that did

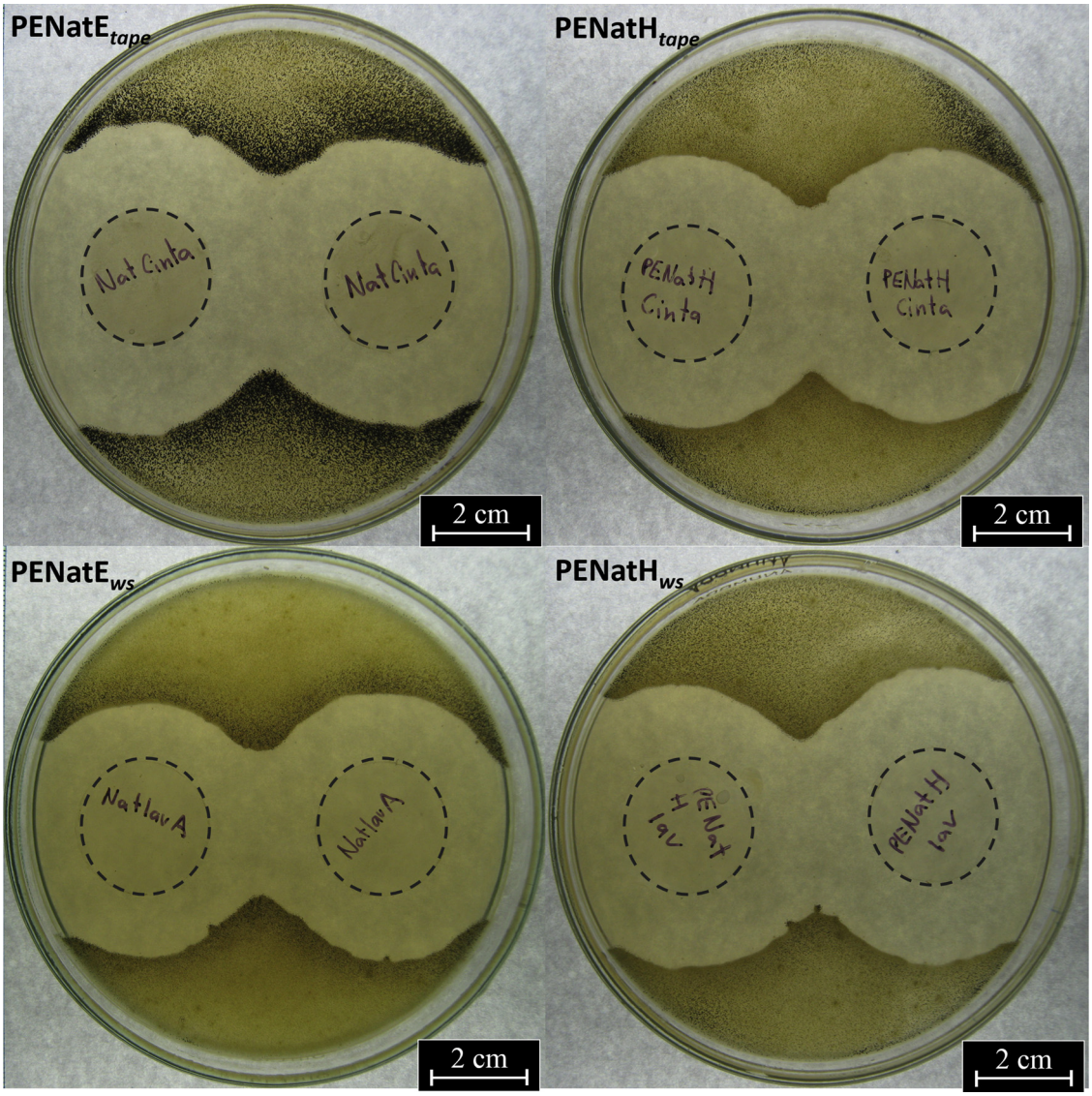

Fig. 8. Inhibition zone of the extracted coated films with tape (PENatE$_{tape}$ and PENatH$_{tape}$) and sonication in water (PENatE$_{ws}$ and PENatH$_{ws}$) against *A. niger*.

not affect its growth, as expected. Both coated films, PENatH and PENatE, showed antifungal activity with very similar inhibition zone diameter aprox. 3 cm, confirming natamycin activity preservation onto it. They inhibited the *A. niger* growth even when their amount is smaller than Nat control. The above discussion confirms that the proposed coating process and the changes in the natamycin crystallinity did not affect either its stability or its activity.

In order to analyze whether modified films activity is preserved even under severe packaging handling conditions, antifungal activity of coated films after extraction treatment with tape and sonication in water was analyzed. Results in Fig. 8 evidence that despite the harshness extraction treatments, antifungal activity of coated films against *A. niger* remain unchanged. It is important to note that there are not significant differences among inhibition zone diameters of coated films and all extracted ones (Figs. 7 and 8). These results agree with the fact that active Nat content onto films surface is greater than 0.015 mg/cm^2, even in the extracted samples. This value was reported by Türe, Eroğlu, Soyer, and Özen, (2008) as the minimal inhibitory dose for *A. niger*. If all the Nat sprayed onto the films had remained active, its concentration would be around of 0.089 mg/cm^2, 6 times more than the minimum dose reported. Antifungal activity is preserved even in PENatH$_{ws}$, where particles were not easily detected (Fig. 5). That is to say, more than 0.015 mg/cm^2 of Nat initial content - representing more than 17% - remains attached to PE film after severe extracting conditions. The last result is very promissory for the final film application targets, antifungal flexible packaging for handling without special care.

Nat coated films from ethanol solution and from n-heptane suspension were antifungal effective against *A. niger*; in fact, the extraction treatments did not affect the films active power. This phenomenon shows a strong adhesion of the particles to the PE matrix, in addition to an excess in the amount of Nat incorporated in the films.

4. Conclusions

A low-cost antifungal film for cheese flexible packaging was obtained by Nat sticking onto PE surface without adhesive use. A versatile process involving spraying of Nat solution and/or suspension onto hot softened PE surface was developed. A good Nat coverage with well adhered particles to PE film either using Nat solution or suspension was obtained, thus demonstrating that both spraying solvents systems are effective. In fact, neither Nat nor PE were degraded during the treatment.

However, PE film coverage morphology is different depending on the used spraying system. Agglomerated particles with similar size and

good distribution onto the whole film surface were obtained when a Nat/ethanol solution was used. These particles presented changes in their crystallinity with respect to initial Nat, but preserve their activity. This fact was interpreted in terms of Nat in-situ crystallization mechanism with insoluble lactose acting as nucleation points onto hot PE surface. On the other hand, when a Nat/n-Heptane suspension was sprayed, a PE coating with relative less homogeneous Nat particle size was achieved, resulting in apparent less surface coverage. This result can be explained considering that particles were sprayed from a suspension, then they suffered attrition, but they did not recrystallize preserving its activity.

In both cases, modified films were active against *A. niger,* even after severe Nat extraction either with tape or with sonication under water. These treatments evidence that particles are well adhered to PE surface confirming that the proposed process to Nat attachment is effective and efficient. In addition, these results mean that modified films can be intensively handled without losing their activity.

The modified films obtained by spraying Nat/ethanol solutions are more sustainable to be used for actual application to cheese antifungal packaging. Ethanol presents greater coverage homogeneity and lower cost than n-heptane. Besides, PENatE does not show detectable solvent residues, if any, it they would be non-toxic. Moreover, the proposed process is easy to use during film production; i.e. in the calendaring step of continuous cast film extrusion.

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