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Controlled Release of Antimicrobial Compounds from Highly Swellable Polymers

G. G. BUONOCORE,[1] M. SINIGAGLIA,[2] M. R. CORBO,[2] A. BEVILACQUA,[2] E. LA NOTTE,[2] AND M. A. DEL NOBILE[2]*

[1]*Institute of Composite and Biomedical Materials, National Research Center P. le Tecchio, 80-80125 Naples, Italy; and* [2]*Department of Food Science, University of Foggia, Via Napoli, 25-71100 Foggia, Italy*

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ABSTRACT

The suitability of antimicrobial release films made from highly swellable polymers for use in food packaging was evaluated. The possibility of modulating the release kinetics of active compounds either by regulating the degree of cross-link of the polymer matrix or by using multilayer structures was addressed. The release kinetics of lysozyme, nisin, and sodium benzoate (active compounds with different molecular weights) were determined at ambient temperature (25°C). The effectiveness of the proposed active films in inhibiting microbial growth was addressed by determining the antimicrobial efficiency of the released active compounds. *Micrococcus lysodeikticus, Alicyclobacillus acidoterrestris,* and *Saccharomyces cerevisiae* were used to test the antimicrobial efficiency of released lysozyme, nisin, and sodium benzoate, respectively. Results indicate that the release kinetics of both lysozyme and nisin can be modulated through the degree of cross-link of the polymer matrix, whereas multilayer structures need to be used to control the release kinetics of sodium benzoate. All the active compounds released from the investigated active films were effective in inhibiting microbial growth.

In recent years, interest in antimicrobial packaging materials has increased considerably because of the development and diffusion of active packaging concepts. These new types of packaging devices are used with the aim of prolonging the shelf life of the packaged foodstuff by reducing the growth rate of spoilage microorganisms. Two different types of antimicrobial systems are available, those release systems for which it is possible to control the release rate of the active compound and those for which the release rate is uncontrolled (i.e., the preservative is immediately released when the film contacts the foodstuff). The aim of controlled release systems is to transfer the antimicrobial agent to the packed food at a specific rate to maintain a predetermined concentration of the active compound in the release medium for a predetermined period of time. For this reason, controlled release systems have been mainly studied and developed for pharmaceutical applications *(1, 3, 4, 7).* Their use in food packaging is still limited. The only study of this application was done by Han and Floros *(6),* who examined the mechanism controlling the release of potassium sorbate from low-density polyethylene into cheese. They suggested the use of a multilayer structure to control the release kinetics: an outer barrier layer, a matrix layer containing the antimicrobial, and a release control layer.

In pharmaceutical applications, the most common polymer matrices used for active compound release devices are highly swellable polymers (i.e., polymers that absorb a large amount of water and swell considerably). To our knowledge, the only study on active agent release from highly swellable polymers in food packaging was conducted by Buonocore et al. *(2)*; all other studies have been restricted to release of antimicrobial agents from hydrophobic or moderately hydrophilic polymer matrices.

The aim of this research was to develop and study antimicrobial films in which the polymer matrix is a highly swellable polymer. The possibility of controlling the release kinetics of active compounds either by controlling the degree of cross-link of the polymer matrix or by using multilayer structures was addressed. The antimicrobial efficiency of the active films also was tested by determining the growth of *Micrococcus lysodeikticus, Alicyclobacillus acidoterrestris,* and *Saccharomyces cerevisiae* in the presence of the active film.

MATERIALS AND METHODS

The films studied were obtained by cross-linking polyvinyl alcohol (PVOH; P1763, Sigma, St. Louis, Mo.). The molecular weight of this compound is 70,000 to 100,000, and it is hot water soluble and has a viscosity (4% water solution at 20°C) of 11 to 14 cps. The cross-linking agent was glyoxal (40% aqueous solution, Sigma-Aldrich, Gallarate, Milan, Italy), and the catalyzer was HCl (37%, Sigma-Aldrich). The active compounds used were lysozyme from chicken egg white (Sigma-Aldrich), nisin (donated by Danisco Innovation, Beaminster, Dorset, UK), and sodium benzoate (Sigma-Aldrich).

Film preparation. PVOH (3.25 g) was dissolved in 25 ml of distilled water by incubating the solution for 30 min in an autoclave at 120°C. The solution was slowly cooled and cross-linked by first adding a known amount of glyoxal during moderate stirring and then immediately adding 0.2 ml of a 37% aqueous solution of HCl. The obtained solution was cast onto a Plexiglas plate and dried under ambient conditions (27°C and 55% humidity) for 48 h. Films had an average thickness of 120 μm. The

* Author for correspondence. Tel: +39 881 589 242; Fax: +39 881 740 211; E-mail: ma.delnobile@unifg.it.

TABLE 1. *Classification of PVOH films*

Active compound	% glyoxal (wt/wt) 7.7	2.0	0.77	0.077
None	A	B	C	D
Lysozyme				
100 mg	A_L	B_L	C_L	D_{L1}
200 mg				D_{L2}
500 mg				D_{L3}
Nisin, 100 mg	A_N	B_N	C_N	D_N
Sodium benzoate, 100 mg	A_{SB}	B_{SB}	C_{SB}	D_{SB}
Multilayer	E_{SB} = D/(non–cross-linked PVOH + 100 mg SB)/D_{SB}			

thickness of the films was measured using a Digimatic Micrometer (Mitutoyo, Milan, Italy; ±0.5 μm). The value of the film thickness was obtained by averaging 100 measurements.

The active films were obtained using the following procedure: PVOH (3.25 g) was dissolved in 25 ml of distilled water by incubating the solution for 30 min in an autoclave at 120°C. The solution was slowly cooled at room temperature, a known amount of active agent was added, and the solution was stirred at ambient temperature until the preservative was completely dissolved. The solution was cross-linked by adding a known amount of glyoxal during moderate stirring and then immediately adding 0.2 ml of a 37% aqueous solution of HCl.

Multilayer active film was produced with three layers: a cross-linked PVOH film, a non–cross-linked PVOH film containing the active compound, and another cross-linked PVOH film. Each layer was made using the same procedure. The films produced and investigated are summarized in Table 1.

Active compound release kinetics. The prepared active films (0.012 cm by 200 cm^2) were put into a container and brought in contact with 250 ml of distilled water at room temperature during moderate stirring. The active compound release kinetics were evaluated by using high-pressure liquid chromatography (HPLC) to monitor the antimicrobial concentration in the surrounding solution until an equilibrium value was reached. Each value reported is the average of three replications.

HPLC active compound assay. Quantitative determination of lysozyme in the water solution was performed following the method proposed by Liao et al. *(8)*. Lysozyme was assayed using HPLC (model 1100, Agilent, Palo Alto, Calif.). A C18 reverse-phase column (250 by 4 mm, 5 μm) and a gradient elution with water-acetonitrile gradients (1 ml/min) containing 0.1% trifluoroacetate were used. A typical gradient was 0 to 60% acetonitrile over 20 min, with lysozyme eluting at 10 min. Detection was at 254 and 225 nm. A calibration curve was constructed for peak area against lysozyme concentration for standard solutions from 6 to 300 ppm, with five replicate samples for each lysozyme concentration.

Quantitative determination of nisin into the water solution was performed following the method proposed by Liu and Hansen *(9)*. Nisin was assayed using HPLC (Agilent). A C18 reverse-phase column (250 by 4 mm, 5 μm) and a gradient elution with water-acetonitrile gradients (1 ml/min) containing 0.1% trifluoroacetate were used. A typical gradient was 20 to 60% acetonitrile over 20 min, with nisin eluting at 10 min. Detection was at 254 and 225 nm. The calibration curve was constructed for peak area against nisin concentration for standard solutions from 2.5 to 100 ppm, with five replicate samples for each nisin concentration.

Quantitative determination of sodium benzoate in water was performed using the method proposed by Pylypiw and Grether *(10)*. Sodium benzoate was assayed using HPLC (Agilent) and a C18 reverse-phase column (250 by 4 mm, 5 μm). Isocratic conditions of mobile phase with a flow of 0.8 ml/min were used, with sodium benzoate eluting at 15 min. Detection was at 225 nm. A calibration curve was constructed for peak area against sodium benzoate concentration for standard solutions from 2.5 to 100 ppm, with five replicate samples for each sodium benzoate concentration.

Evaluation of antimicrobial activity of released active compounds. The antimicrobial effect of films containing lysozyme on viable *Micrococcus lysodeikticus* cells was determined by transferring a loopful of culture from nutrient agar (Oxoid, Milan, Italy) slants to 50 ml of nutrient broth (Oxoid) and incubating for 24 h at 30°C. Five milliliters of nutrient broth was diluted twofold in the same medium. Absorbance (450 nm) of the dilutions was measured, and 0.1 ml of each dilution was enumerated using the spread plate method. A standard curve of CFU per milliliter versus absorbance was obtained and used to estimate the initial inoculum size.

D_{L1}, D_{L2}, and D_{L3} sterile films (Table 1) containing 100, 200, and 500 mg of lysozyme, respectively, were placed into 100 ml of nutrient broth inoculated with approximately 10^7 CFU/ml of *M. lysodeikticus*. The samples were incubated at 30°C, and 1-ml aliquots were removed at 0, 2, 4, 6, 9, and 24 h. The samples were diluted in sterile saline solution (0.9% NaCl) and enumerated in nutrient agar. Film D, which did not contain lysozyme, served as a control.

Effectiveness of film containing nisin against *A. acidoterrestris* spores was determined by inhibition zone assays. Sporulation was achieved on acidified malt extract agar (Oxoid); the pH of the medium was adjusted to 4.5 with a solution of citric acid 1:1 (wt/wt). After incubation for 3 to 5 days at 45°C, spores were washed from the surface of the agar with cold sterile distilled water and centrifuged at 3,000 × *g* for 15 min. The pellet was washed three times and then suspended in sterile distilled water. To destroy the vegetative cells, the spore suspension was heat shocked for 10 min at 80°C. The number of spores was determined by microscopy and after plate counting on acidified malt extract agar.

An inhibition zone assay was conducted by inoculating acidified malt extract agar with 1 ml of a suspension of spores (10^6 CFU/ml). For free nisin tests, 0.1 ml of a nisin solution (300 IU/ml) was poured into wells (7 mm diameter) previously cut into the agar medium. For films, 0.05 g (18 by 18 mm) containing

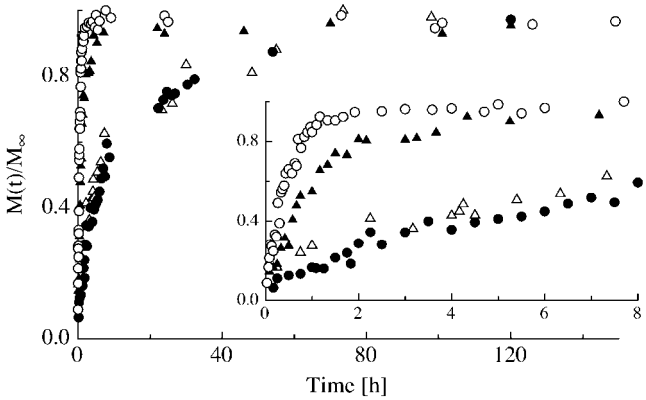

FIGURE 1. *Normalized release kinetics of lysozyme in water at 25°C. △, Film A_L; ●, film B_L; ▲, film C_L; ○, film D_L. Inset: magnification of the first part of the graph.* M(t) *is the quantity of lysozyme released at time* t*;* M_∞ *is the value of* M(t) *at equilibrium.*

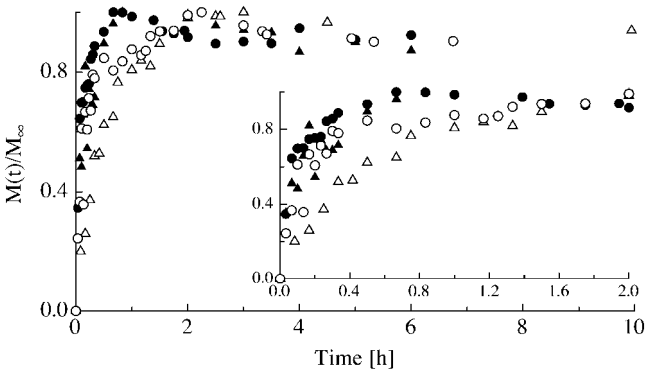

FIGURE 3. *Normalized release kinetics of sodium benzoate in water at 25°C. △, Film A_{SB}; ●, film B_{SB}; ▲, film C_{SB}; ○, film D_{SB}. Inset: magnification of the first part of the graph.* M(t) *is the quantity of sodium benzoate released at time* t*;* M_∞ *is the value of* M(t) *at equilibrium.*

about 300 IU of nisin were cut from A_N and D_N films and placed onto appropriately inoculated agar in petri dishes.

After incubation at 44°C for 48 h, the inhibition zones were measured to the nearest 1 mm; all the experiments were performed in triplicate.

Saccharomyces cerevisiae 71B (DAL CIN S.p.A, Milan, Italy) was maintained on Sabouraud dextrose agar (Oxoid) slants at 4°C. A loopful of culture was transferred to 10 ml of acidified Sabouraud broth (Oxoid) and incubated at 25°C for 48 h; the pH of the medium was adjusted to 4.5 with a solution of citric acid (1:1, wt/wt). Dilutions of yeast suspension were made in sterile saline solution (0.9% NaCl) to obtain about 10^5 CFU/ml, and 1 ml of this suspension was transferred into 100 ml of acidified Sabouraud broth (pH 4.5) containing 100 mg of sodium benzoate and into 100 ml of acidified Sabouraud broth containing film D_{SB} with 100 mg of sodium benzoate. Samples were incubated at 25°C, and 1-ml aliquots were removed after 24 h. The samples were diluted in 0.9% sterile saline solution and enumerated in Sabouraud dextrose agar. Film D, which did not contain benzoate, and a culture of viable cells in Sabouraud broth served as controls.

RESULTS AND DISCUSSION

An active packaging system based on the release of antimicrobial compounds must fulfill two main requirements: the release kinetics of the antimicrobial agent should be adjustable to the specific requirements of the packaged food and the released active compound should maintain a certain antimicrobial efficiency. We discuss separately these two aspects, which are of great relevance in the development of antimicrobial systems intended for food packaging applications.

Active compound release kinetics. The normalized release kinetics of lysozyme, nisin, and sodium benzoate in water from PVOH films at 25°C differed in the degree of cross-link (Figs. 1 through 3). The normalized release kinetics in these figures were obtained by plotting the ratio of the amount of active compound released at time t, $M(t)$, to the total amount of active compound release at equilibrium, M_∞, as a function of time. For lysozyme and nisin, increasing the degree of cross-link of PVOH increases the time required to reach equilibrium conditions, suggesting that the release kinetics of these compounds can be adjusted by acting on the degree of cross-link. In contrast, the degree of cross-link did not influence the release kinetics of sodium benzoate, indicating that the molecular size of sodium

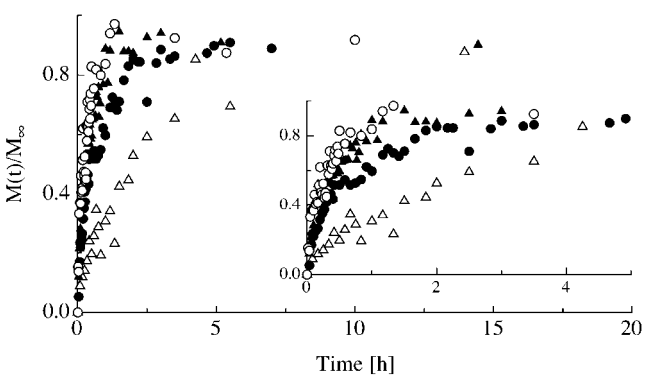

FIGURE 2. *Normalized release kinetics of nisin in water at 25°C. △, Film A_N; ●, film B_N; ▲, film C_N; ○, film D_N. Inset: magnification of the first part of the graph.* M(t) *is the quantity of nisin released at time* t*;* M_∞ *is the value of* M(t) *at equilibrium.*

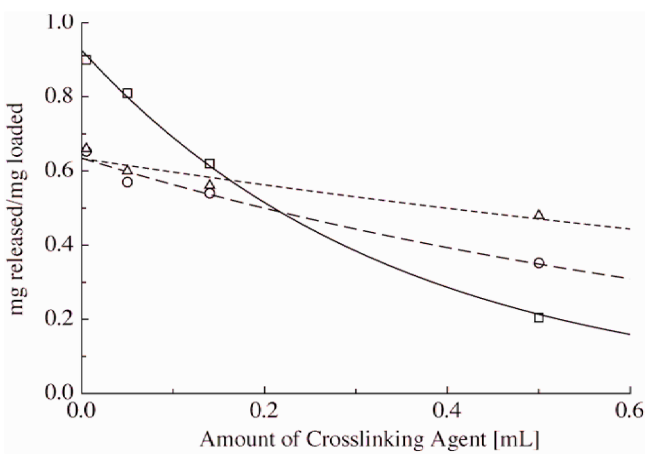

FIGURE 4. *Ratio of the amount of antimicrobial agent released at equilibrium to the amount of antimicrobial agent loaded into the film, plotted as a function of the cross-linking agent concentration. □, Lysozyme; ○, nisin; △, sodium benzoate.*

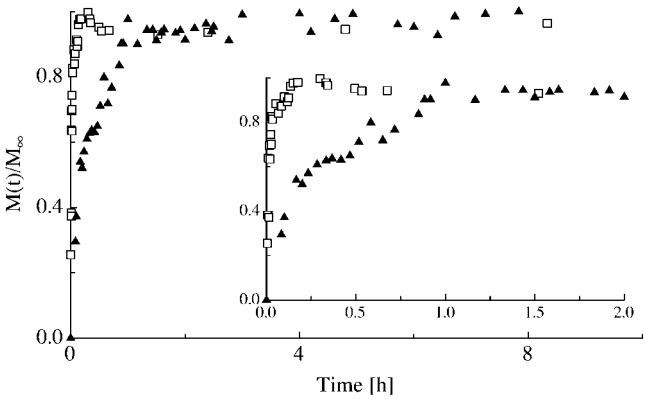

FIGURE 5. *Normalized release kinetics of sodium benzoate in water at 25°C.* □, *Film* D_{SB}; ▲, *film* E_{SB}. *Inset: magnification of the first part of the graph.* M(t) *is the quantity of sodium benzoate released at time* t; M_∞ *is the value of* M(t) *at equilibrium.*

benzoate is smaller than the mesh size of the investigated cross-linked PVOH films.

Figure 4 illustrates the ratio of the amount of antimicrobial agent released at equilibrium to the total amount of antimicrobial agent loaded in the film plotted as a function of degree of cross-link. The degree of cross-link was expressed as the amount of cross-linking agent used (the curves shown in the figure do not represent any particular model; they have been drawn to highlight the trend in the data). The amount of the active compound released at equilibrium always decreased as the degree of cross-link of the polymer matrix increased. Considering that the degree of cross-link slightly influences the partition coefficient of the active agent, we hypothesized that part of the preservative loaded into the film is chemically bonded to the polymer backbone via the cross-linking reaction. One of the two active sites of the cross-linking agent could react with one of the sites on the polymer backbone, attaching the cross-linking agent to the matrix, whereas the other site could react with the active compound, bonding the preservative to the matrix. Therefore, as the quantity of the cross-linking agent increases, the number of sites should increase, leading to an increase in the number of active compound molecules bonded to the PVOH backbone.

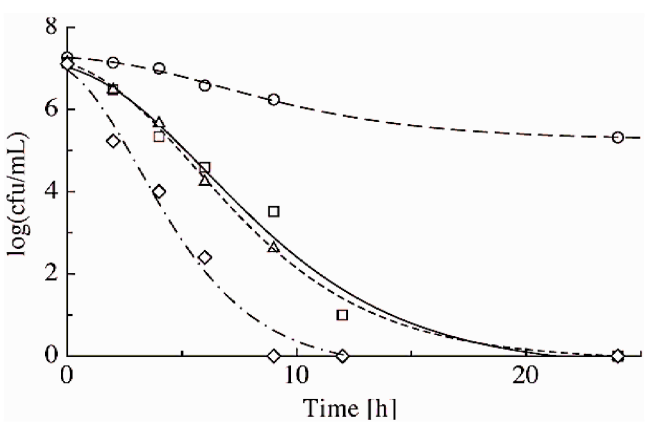

FIGURE 6. *Evolution of* Micrococcus lysodeikticus *cell loads in the presence of films with and without lysozyme.* ○, *Film D;* □, *film* D_{L1}; △, *film* D_{L2}; ◇, *film* D_{L3}.

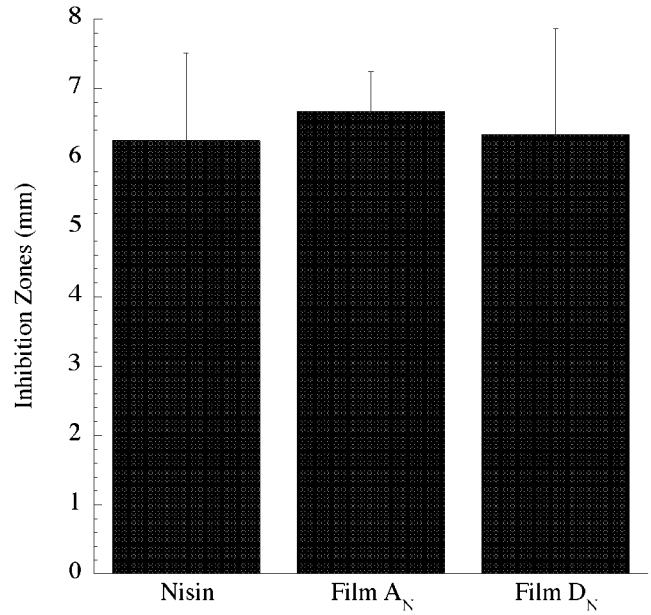

FIGURE 7. *Inhibition zones of* Alicyclobacillus acidoterrestris *grown in the presence of both pure nisin and active films.*

It is not possible to control the release kinetics of sodium benzoate through the degree of cross-link of the polymeric matrix (Fig. 3). The use of a multilayer structure is a possible way to overcome this problem and control the release kinetics of small molecules from highly hydrophilic matrices. Figure 5 illustrates the normalized release kinetics of films D_{SB} and E_{SB}. The introduction of a diffusive resistance appeared to be effective in slowing down the release kinetics of small molecules, such as sodium benzoate, from hydrophilic polymers.

Antimicrobial efficiency of released compounds. *M. lysodeikticus* grown in nutrient agar was used to test the antimicrobial efficiency of lysozyme released from the investigated active films. Figure 6 illustrates the effect of the presence of both films loaded and unloaded with the antimicrobial compound (the curves shown do not represent

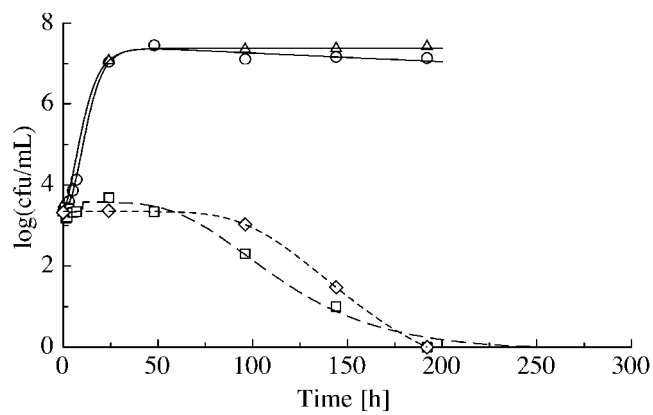

FIGURE 8. *Evolution of* Saccharomyces cerevisiae *cell loads grown in the presence and absence of pure sodium benzoate and in the presence of films with and without sodium benzoate.* ○, *Absence of active film;* □, *pure sodium benzoate;* △, *film D;* ◇, *film* D_{SB}.

any particular model; they have been drawn to highlight the trend in the data) on cell survival. There is only a slight decrease in the cell concentration in film D (control); whereas when the test microorganism was exposed to the active films, there was a marked decrease in microbial population (ca. 7 log cycles with respect to the inoculum). This finding suggests that the lysozyme released from the films retained its antimicrobial efficiency and had the potential to reduce bacterial populations.

The antimicrobial efficiency of nisin released from the active films was tested on *A. acidoterrestris* spores. These spores resist both high temperatures and acidic environments *(5)*; therefore, this organism has become a potential spoilage concern in hot-fill fruit and vegetable juices. Preliminary experiments in solid medium revealed that sensitivity of *A. acidoterrestris* to nisin was detected at 60 IU. However, to obtain a detectable inhibition zone diameter (of about 5 mm) it was necessary to use a higher concentration. As in the case of lysozyme, results suggest that released nisin does not lose its efficiency as a antimicrobial agent (Fig. 7). The inhibition zone diameters of the released nisin for both film A_N and film D_N are similar to that of pure compound.

Saccharomyces cerevisiae grown in Sabouraud dextrose agar was used to test the antimicrobial efficacy of sodium benzoate released from the active films. Figure 8 illustrates the effect on these yeast cells of films with and without sodium benzoate. Sodium benzoate released from the antimicrobial films retained the activity of the pure compound and was effective in inhibiting yeast growth (the curves shown in the figure do not represent any particular model; they have been drawn to highlight the trend in the data). It is still necessary to determine whether significant effects can be achieved in foods; because the composition of foods and storage temperatures are variable, the results here cannot be generalized.

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