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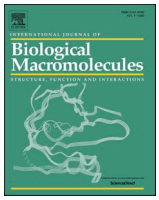

Optimisation of stimuli-responsive films based on dynamic α,β-unsaturated imines from chitosan and trans-2-hexenal to enhance the antimicrobial acid-response

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ABSTRACT

Trans-2-hexenal (HX) is a potent antimicrobial which can be reversibly stabilised in chitosan (CS) films forming α,β-unsaturated imines. The hydrolysis of the imines promoted by acid environments triggers the release of HX to the media exerting its antimicrobial activity. It is known that besides imines, the electrophilic β-alkene carbon of HX can form Michael adducts with primary amino groups of chitosan. However, the formation of nucleophile-C bonds is undesired since these bonds are barely hydrolysed and limit the release of HX and by hence, the effectivity of the film. Thus, the aim of this work has been to optimise the formation of trans-2-hexenal-imine-chitosan films employing response surface methodology in order to favour the formation of conjugated imines avoiding Michael adducts. The optimisation of the reaction parameters indicated that synthesis temperature of 10 °C and without the use of an acid catalyst favours the formation of conjugated imines. Spectroscopic techniques, elemental analysis and swelling behaviour in various media were used to characterise the optimised films. The release kinetics of HX and the antimicrobial activity of the films were also studied. The present work provided relevant information to increase the antimicrobial efficacy of trans-2-hexenal-imine-chitosan films for the development of active food packaging.

1. Introduction

Naturally-occurring volatiles with antimicrobial capacity have been widely used in the development of antimicrobial active packaging for postharvest horticultural produce. This active packaging system is based on the sustained release of volatiles from the packaging system into the headspace of the package for finally reaching the surface of the fresh produce. Commonly, the volatile is physically entrapped in a polymer matrix forming part of the packaging system. However, this technology has several drawbacks when employing volatiles such as their loss during the processing and storage of the active material. Moreover, there is a lack of smart mechanisms for the triggering of the active volatiles when is required. Several strategies have been employed with the aim to reduce the rapid loss of the volatile from the polymer, such as a previous encapsulation of the volatile in cyclodextrins or biopolymeric matrices, and more recently in sophisticated materials, such as mesoporous silica and metal organic frameworks (MOFs) [1–5]. A new approach to develop active materials that release antimicrobial volatiles is their stabilisation in the polymer matrix through the formation of reversible covalent bonds. The main feature of this type of bonds is their responsiveness under certain external stimuli [6]. Thus, reversible covalent bonds can be used to anchor active molecules in a polymer and trigger their release when is needed by the action of pH, temperature, light, etc. that promote the cleavage of the bond [7–9]. Imines are particularly attractive for the development of these responsive polymers. The imine bond (C=N) is formed through the condensation reaction of carbonyl group of aldehydes with primary amino group, and normally requiring acidic activation (protic or metallic) of the corresponding carbonyl compound [10]. Treatment of imines with mildly acidic water leads to their rapid hydrolysis to give the starting original amino and aldehyde groups.

Due to the abundance of primary amino groups in chitosan, this biopolymer is a potent candidate for the design of smart responsive antimicrobial films. These films are based on the reversible covalent

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grafting of human safe antimicrobial aldehydes to primary amino groups of chitosan forming imines. In this line, trans-2-hexenal (HX) is a green leaf volatile released by stressed or tissue injured plants to protect them against fungi and insects [11,12]. This aldehyde is classified as safe for human consumption and commonly used as a flavouring agent [13]. Due to the ability of HX to fight microbial infection, this volatile molecule is being studied as an ecological alternative to synthetic fungicides and to control postharvest decay of fresh produce [14,15].

HX is difficult to handle when is used in active packaging due to its high volatility. In order to maintain the stability of HX during prolonged storage and before its use, our group has recently conducted studies on its stabilisation in chitosan films via the formation of reversible imines [7,16]; the hydrolysis of the imine which can be promote via pH allows the release of HX from the films. However, HX has a double bond conjugated with the carbonyl, and besides the imine formation through reaction with the primary amino groups of chitosan, these groups can react with the electrophilic β-alkene carbon forming Michael adducts hindering the release of HX; moreover, the carbonyl of the Michael adduct could form imines with other amino groups of chitosan causing the cross-linking of the matrix and hindering the release of HX and the efficiency of the antimicrobial pH-responsive system.

On the basis of the above, the main purpose of this study has been to optimise the development of responsive trans-2-hexenal-imine-chitosan films avoiding the formation of Michael adducts. For that, CS:HX weight ratio, temperature, and the use of hydrochloric acid (HCl) as a catalyst were optimised by employing response surface methodology (RSM) in order to obtain films with a high content of conjugated imines and thus, with adequate release of HX. Optimised films were characterised by spectroscopic techniques, elemental analysis and swelling behaviour. The release kinetics of trans-2-hevexal from optimised films was studied and modelled, and the antimicrobial efficacy of the films against *Penicilium expansum* and *Escherichia coli* was evaluated.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (deacetylation degree 75–85 % and molecular weight range of 50–190 kDa), trans-2-hexenal, disodium phosphate and citric acid monohydrate, used to prepare buffer solutions at pH 7 and pH 3, were obtained by Sigma-Aldrich (Barcelona, Spain). Sodium hydroxide (NaOH), ethanol 96 % *(v/v)* and hydrochloric acid 37 % (HCl), were obtained by Scharlab (Barcelona, Spain). *Escherichia coli* and *Penicillium expansum* were provided by the Spanish Type Culture Collection (CECT 434, CECT 2278, respectively). The growth media for microorganism, Tryptone Soy Broth (TSB), Tryptone Soy Agar (TSA) and Potato Dextrose Agar (PDA) were purchased from Scharlab (Barcelona, Spain).

2.2. Functionalisation of chitosan films with trans-2-hexenal

Chitosan films were prepared using the casting method by dissolving 1.5 g of low molecular weight chitosan in 100 mL of a 0.5 % *(v/v)* aqueous acetic acid solution. The acetate chitosan solution was stirred at 50 °C until dissolved. The solution was then filtered and casted on polystyrene plates and dried at 37 °C for 24 h. The dried acetate chitosan films with a thickness of 35 ± 5 μm were neutralised with a 0.1 M NaOH solution for 24 h. After that, the films were washed repeatedly with deionised water. Neutralised chitosan films (CS) were employed for aldehyde immobilisation on the backbone chitosan polymer. The reaction parameters: temperature (x_1), HCl concentration (x_2) and the aldehyde concentration (x_3) will be further detailed in *Section 2.3*.

The reaction occurred at the solid (film) and liquid (aldehyde) interface using ethanol as the solvent reaction. For this purpose, 2 g of chitosan film were cut in 1.5 × 1.5 cm^2 squares and added in 75 mL of ethanol 96 % (*v/v*) where trans-2-hexenal (x_3) was previously dissolved. Different amount of HCl (x_2) were then added as a catalyst. The flask was placed in a shaking bath at constant temperature (x_1) for 24 h. To remove unreacted aldehyde, CS films were then extracted from the reaction medium and washed for 24 h with ethanol 96 % *(v/v)* in a shaking bath at the reaction temperature. Finally, control CS and modified-chitosan (CSHX) films were dried and stored in a desiccator with silica gel to use.

2.3. Response surface methodology (RSM) modelling, optimisation and validation

2.3.1. Box-Behnken experimental design

The influence of some parameters linked to the reaction between the carbonyl group of the aldehyde and the primary amino groups of chitosan owing to chitosan-functionalisation was evaluated. A response surface model of three relevant reaction parameters was carried out in order to obtain CSHX films with the best trans-2-hexenal release response. The experimental design and statistical analyses were performed by using Statgraphics Centurion XVIII software (StatPoint Technologies Inc., USA) according to Box-Behnken (BB) design. BB experimental design based in a 3-level-3-factor required a total of 15 experimental runs, which included three replicates at the central point of the factors. The effect of the reaction temperature, the amount of trans-2-hexenal, and the pH of the reaction determined by HCl added to the reaction medium was studied on the chemical immobilisation of HX in chitosan films, and the parameters were optimised to obtain the maximum release of HX when the imine of functionalised film was hydrolysed. Three factors were assessed, and their levels (x_i) were: temperature (10 and 60 °C), trans-2-hexenal quantity respect to 2 g of chitosan films (0.5 and 2 g), amount of HCl (0 and 100 μL), each variable was tested at three different coded levels (X_i), as high (+1), medium (0), low (−1). Table 1 shows the experimental design generated.

A second-degree polynomial was used to fit the measured responses to the coded variables as shown in the following Eq. (1):

$$Y_n = \beta_0 + \sum_{i=1}^{2}\beta_i X_i + \sum_{i=1}^{2}\sum_{j=2.j>i}^{3}\beta_{ij} X_i X_j + \sum_{i=1}^{3}\beta_{ii} X_i^2 \quad (1)$$

where, Y_n was the adjusted predicted response, β_0 was the model constant, β_i was the linear coefficient, β_{ii} was the quadratic coefficient, β_{ij} was the coefficient for the interaction effect, and X_i was a dimensionless coded value of the independent variable x_i. The response surface was estimated from data with a significance of $p < 0.05$.

Table 1
Box-Behnken experimental design matrix and experimentally average values of release obtained.

Run	Factors			Coded variables			Response variable
	x_1 Temperature (°C)	x_2HCl (μL)	x_3 Trans-2-Hexenal (g)	X_1	X_2	X_3	Y; Release (mg/L_{air}·g_{film})
1	10	0	1.25	−1	−1	0	37.0 ± 0.6
2	60	0	1.25	+1	−1	0	8.9 ± 0.8
3	10	100	1.25	−1	+1	0	13.9 ± 0.8
4	60	100	1.25	+1	+1	0	5.7 ± 0.4
5	10	50	0.5	−1	0	−1	15.7 ± 0.6
6	60	50	0.5	+1	0	−1	3.4 ± 0.5
7	10	50	2	−1	0	+1	25.0 ± 2.0
8	60	50	2	+1	0	+1	11.9 ± 0.7
9	35	0	0.5	0	−1	−1	22.0 ± 0.7
10	35	100	0.5	0	+1	−1	9.6 ± 0.6
11	35	0	2	0	−1	+1	38.8 ± 0.7
12	35	100	2	0	+1	+1	17.0 ± 1.0
13	35	50	1.25	0	0	0	21.4 ± 0.6
14	35	50	1.25	0	0	0	20.4 ± 0.3
15	35	50	1.25	0	0	0	22.9 ± 0.8

2.3.2. Response variable: quantification of aldehyde released by gas chromatography

The trans-2-hexenal released from the functionalised chitosan films was selected as the response variable. The reversibility of imine bond formed between trans-2-hexenal and primary amino groups of chitosan was evaluated by immersing the functionalised films into a buffer solution adjusted at pH 3, being sufficiently acidic to promote the rapid hydrolysis of the reversible imine bond and, subsequently, the aldehyde release. For that purpose, 50 mg of films were placed in a 20 mL vial and covered with 5 mL of the buffer solution at pH 3. To collect the 1 mL headspace sample, the vials were sealed with a crimp cap fitted with a septum. The assay was performed at 23 °C and the trans-2-hexenal release was measured after 2 h of film immersion.

HX release was measured by gas chromatography (GC) using a module 6850 Series II Network GC System (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionisation detector (FID) and a Restek RTX1 capillary column (30 m length × 0.53 mm diameter × 5 μL thickness). The chromatography method was programmed in splitless mode using Helium as carrier gas. Injector and detector temperatures were set at 220 °C. The temperature program was from 75 until 220 °C. A calibration curve was previously established using the chromatographic conditions described above with a known amount of trans-2-hexenal. The results were reported as $mg/L_{air} \cdot g_{film}$ and the analyses were carried out in triplicate.

2.3.3. Validation

The obtained optimal conditions for the synthesis of trans-2-hexenal-imine chitosan films with maximum aldehyde release were employed to validate the model developed. HX release was performed for the optimised films (CSHX) following the procedure detailed in Section 2.3.2. A Student's *t*-test was performed using the value obtained by the model as the reference value.

2.4. Characterisation of optimised CSHX films

After obtaining the optimal conditions for the synthesis of CSHX films, they were characterised. CSHX films with lower weight ratios of CS:HX, 2:1 and 2:0.5 were also studied. Therefore, the properties of three different films CSHX-R_A (2:2 optimised), -R_B (2:1) and -R_C (2:0.5) were evaluated. The aim of this experiment was to study the effect of the amount of HX added to the reaction medium on the formation of Michael adducts when fixing the temperature and the concentration of catalyst at optimal conditions for the formation of conjugated imines, and thus, maximum release of HX.

2.4.1. Chemical characterisation and quantification of aldehyde in CSHX films

The chemical modification of chitosan with trans-2-hexenal was assessed using solid-state ^{13}C nuclear magnetic resonance (^{13}C NMR) on CSHX films. A Bruker DSX 400 spectrometer, operating at resonance frequencies of 399.53 MHz for ^{1}H and 100.47 MHz for ^{13}C, was employed for the spectral acquisition. The ^{13}C CP/MAS spectra were recorded with a 3.9-second 90° pulse, a 3-second pulse delay time, an acquisition time of 30 ms, and 2000 scans. All NMR spectra were obtained at 300 K using broadband proton decoupling and a standard cross-polarization pulse sequence. To minimize resonance broadening caused by the anisotropy of chemical shift tensors, an MAS rate of 7.0 kHz was employed.

Degree of substitution (DS, %) was determined using the methodology proposed by Inukai et al. [17] to quantify the trans-2-hexenal chemically incorporated in chitosan films. For this purpose, C/N ratio was obtained by elemental analysis of CSHX films using a CHN elemental analyser (CE Instruments EA 1110, Thermo Fisher Scientific, Waltham, MA, USA). The degree of acetylation (DA, %) of chitosan was also calculated according to a previous reported methodology [18]. In addition, the degree of substitution was also determined for all experimental runs as a reference. Three replicates were analysed for each sample.

2.4.2. Swelling and stability of CSHX films

The degree of swelling of optimised CSHX films and the stability of the imine bond was assessed at neutral and acidic pH. To achieve this, the films were immersed in glass vials with a buffer medium fixed at pH 3 and pH 7 and they were stored for 21 days at 23 °C. Film samples were taken on days 1, 3, 7, 14 and 21 and the water uptake (WU) was calculated with the following Eq. (2):

$$WU(\%) = \frac{(M_{wet} - M_{dry})}{M_{dry}} x100 \quad (2)$$

where M_{wet} is the mass of the film after immersion in the buffer and M_{dry} is the mass of the film after drying. Furthermore, the swelling was determined for all experimental runs to serve as a reference. Three replicates were analysed for each sample.

2.4.3. Kinetics of trans-2-hexenal released from CSHX films

Relative cumulative release profiles of trans-2-hexenal (HX) from optimised films were measured using a desorption system coupled to a gas chromatograph, and using different buffer solutions fixed at pH 3 and 7. To achieve this, a film sample with a weight around 15 mg and size of 1.5 × 1.5 cm^2 was placed in a desorption vial, which allowed the incorporation of 5 mL of the buffer solution to trigger the imine bond hydrolysis and the volatile compound release. The vial allowed the entrance of 10 mL/min of humid air flux to drag the released aldehyde vapour into the gas chromatograph injector. The concentration of aldehyde in the flow was quantified using an Agilent 7890 GC System (Agilent Technologies, Palo Alto, CA, USA) with a 250 μL automatic injection valve. The GC was equipped with a flame ionisation detector (FID) and an Agilent HP-5 column (30 m, 320 μm of diameter and 0.25 μm of thickness) with a flow rate of 12 mL/min and helium as carrier gas. Thermal conditions were as follow: 250 °C of FID and 230 °C for injector, splitless injection, valve box temperature at 150 °C and the oven temperature had a gradient from 40 to 240 °C in a time of 15.33 min. A calibration curve was established in advance by injecting known amounts of HX.

2.5. Antimicrobial assays

The antimicrobial pH-response of optimised films was assayed in vitro against *Penicillium expansum* and *Escherichia coli* using as triggering agent buffer solution at pH 3, and 7.

2.5.1. Microorganisms and inoculum preparation

The microbial strains *P. expansum* and *E. coli* were cultured and maintained in agar-based media. The fungal strains were maintained on PDA at 26 °C by sub-culturing every 7 days. For each strain, a conidial suspension of 10^6 conidia/mL was prepared for the antifungal assay. The fungal surface on the PDA plate was spread with a Digralsky handle after application of sterile peptone water containing Tween 80 (0.05 %). The suspension was transferred to a sterile tube, and serial dilutions were made until a concentration of 10^6 conidia/mL was achieved. An improved Neubauer chamber (Bright-Line Hemacytometer, Hausser Scientific, Horsham, PA, USA) was used for spore count. Bacterial strain was stored at −80 °C in TSB supplemented with 20 % glycerol. Prior to use, the bacterial strain was thawed and transferred on TSA plates at 4 °C. The *E. coli* strain was subcultured on fresh agar monthly. A loopful of *E. coli* from the agar plate was transferred to 10 mL of sterile TSB prior to testing and incubated overnight at 37 °C to obtain bacteria in the stationary phase.

2.5.2. In vitro antimicrobial activity of free trans-2-hexenal

Before evaluating the antimicrobial activity of the films, antimicro-

bial capacity of trans-2-hexenal in vapour phase was evaluated against *P. expansum* and *E. coli*. For that, the Minimal Inhibitory Concentration (MIC) and Minimal Microbicide Concentration (MMC) parameters were determined. The MIC and MMC were defined as the minimum amount of volatile that inhibited microbial growth by at least 50 % and 100 %, respectively, compared to the control. Parameters were expressed as volume of trans-2-hexenal (μL) per volume of air in the Petri dish (cm^3). Petri plates (∅ = 9 cm) containing 15 mL of PDA were inoculated at three equidistant points on the agar surface with 3 μL of fungal suspension (10^6 conidia/mL) for *P. expansum*. In the case of bacterial strain, the overnight culture was diluted to achieve an inoculum concentration of 10^5 CFU/mL. Subsequently, 100 μL of the *E. coli* inoculum was spread on TSA plates (∅ = 9 cm). In both microorganisms, different doses of HX from 0.5 to 20 μL per plate were applied to a sterile paper disc placed on the plate lid. To minimize the loss of volatiles, the plates were closed and sealed with parafilm.

The inoculated plates with *P. expansum* were incubated for 10 days at 26 °C. After seven days of incubation, radial colony growth (cm) was measured to determine the MIC. The aldehyde was then removed from the plate and incubated for a further 3 days, and *P. expansum* growth was evaluated after 10 days to determine the MMC. The bacterial strain was incubated at 37 °C for 24 h, after which the diameter of the inhibition zone was measured to determine MIC and MMC.

2.5.3. Antimicrobial activity of CSHX films

Antimicrobial assays were performed using a double plate system, following the methodology previously described [19]. Briefly, a petri plate with 58 mm of diameter containing agar culture medium, was placed in a petri plate of 90 mm containing the film, and covered with the buffer solution. For *P. expansum*, 3 μL of the conidial suspension (10^6 conidia/mL) was inoculated at the midpoint of the PDA culture medium, and 7.5 mg of film was placed inside the larger plate. A control was carried out without film. The plates were incubated for 10 days at 26 °C. The colony diameter (cm) of *P. expansum* was measured on days 3, 5, 7, and 10 of incubation, and the inhibition percentage was calculated by comparing the results to the control. For the assay with *E. coli*, 60 μL of 10^5 CFU/mL solution was spilled onto TSA plate and spread with a Digralsky handle. The amount of film used in this case was 50 mg. Finally, the plates were incubated at 37 °C for 24 h. After that, the inhibition zone on the agar surface was evaluated. All the tests were conducted in triplicate.

3. Results and discussion

3.1. Response surface methodology (RSM) modelling, optimisation and validation

3.1.1. Box-Behnken experimental design

In the present study, trans-2-hexenal has been employed as the electrophilic substrate for the nucleophilic addition of primary amino groups of chitosan to the carbonyl carbon of the aldehyde also called 1,2 addition, with the aim to form antimicrobial acid-responsive chitosan films functionalised with imines of trans-2-kexenal. Thus, the objective is to obtain the maximum amount of reversible conjugated imines as shown product 1 of Fig. 1. However, the conjugated system of trans-2-hexenal transfers the electrophilic character of the carbonyl to the β-carbon of the double bond, as the electronegative oxygen atom of the carbonyl pulls the electrons away from the β-carbon making it more electrophilic than a typical alkene carbon. Therefore, trans-2-hexenal also can experience conjugated addition of primary amino groups of chitosan or 1,4 addition, forming Michael adducts similar to product 2 of Fig. 1. Michael adducts limits the release of trans-2-hexenal to the headspace of the package and by hence, the effectivity of the film. Fig. 1 also shows the carbonyl of the Michael adduct also can react with new primary amines behaving giving rise to product 3 and crosslinking chitosan chains.

The crosslinking also hinders the release of trans-2-hexenal, which reduces the effectiveness of the responsive film. Our findings demonstrate the importance of experimental reaction parameters such as temperature, the use of an acid catalyst, concentration of aldehyde, etc. in obtaining mostly 1,2 or 1,4 addition products. In general, and based on the bibliography, 1,2-addition products are kinetically preferred, but due to the reversibility of the 1,2-addition step, 1,4-addition products are thermodynamically obtained at high temperatures. With regard to the use of an acid catalyst, the formation of imine bonds between primary amino groups of chitosan and carbonyl groups of trans-2-hexenal can proceed in neutral media although acid media increase the rate of the reaction. However, it has been reported that although the conjugated addition of weak nucleophiles such as the primary amine groups of chitosan to Michael acceptors can sometimes proceed without a catalyst, activation of the conjugated system is required, for example by the presence of H+, despite the protonation of the amines [21]. Regarding our system, it must be taken into account that the primary amino groups

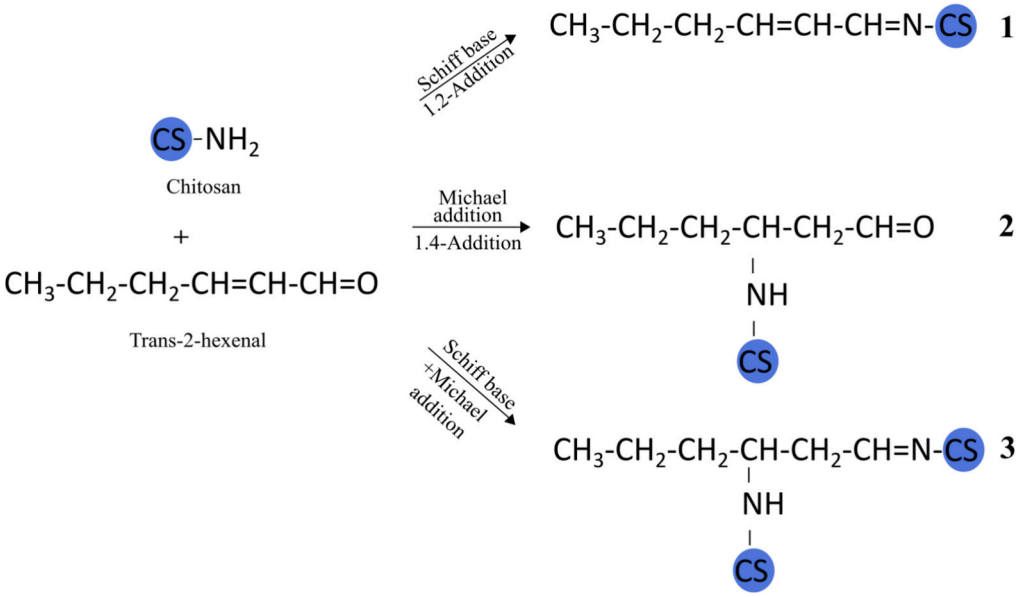

Fig. 1. Scheme of reactions between chitosan and trans-2-hexenal.

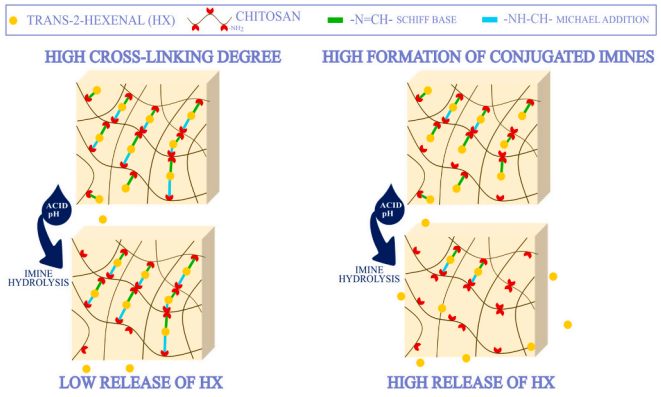

Fig. 2. Scheme showing the dual functionality of trans-2-hexenal as cross-linker or as an active component of pH-responsive antimicrobial films.

of chitosan are in excess and therefore, although some of the groups lose their nucleophilicity when the reaction proceeds with HCl as catalyst, some unprotonated primary amino groups are available for addition.

It is worthy to mention the possibility of oxa-Michael addition in our system, but Michael addition of hydroxyl groups of chitosan to trans-2-hexenal has not been observed. These transformations are typically very challenging due to the low reactivity of the alcohols [22].

Therefore, it is of great interest to develop pH-responsive antimicrobial films to study the reaction parameters to optimise the synthesis of conjugated imines and decrease the formation of Michael adducts and crosslinking as illustrated in Fig. 2.

Table 1 shows the design arrangement for the composition of the reaction parameters for the synthesis of modified films and the experimental results of the dependent variable in the different films.

The results of the response variable (Y) were correctly adjusted using a quadratic model (R2 > 0.97), as expressed by Eq. (3):

$$Y = 975.071 + 11.9307X_1 - 13.4941X_2 + 752.924X_3 - 0.525885X_1^2 + 0.199406X_1X_2 - 0.336267X_1X_3 + 0.0281177X_2^2 - 3.08753X_2X_3 - 95.4104X_3^2 \quad (3)$$

The contributions of the factors that influenced in the response variables were evaluated by ANOVA ($p > 0.05$) and are presented in Table 2. The factors with high contribution on the aldehyde release were in order of significance ($p < 0.05$): Temperature (A) > HCl (B) > amount of trans-2-hexenal (C) > quadratic interaction of temperature (AA) and interaction of temperature and amount of HCl (AB).

To attain a deeper comprehension of how these factors impacted on HX released by the films, response surface plots were also graphed and plotted in Fig. 3. In general, the results showed that the highest release of HX occurred when the reaction was carried out using the greatest concentration of HX, the lowest reaction temperature, and in the absence of a catalyst.

Table S1 in Supplementary Information gives the values of DS for all the runs, DS is related to the amount of HX chemically incorporated to the film during its functionalisation. However, this parameter does not provide information on the kind of nucleophilic addition to HX. It can be 1,2 addition to the carbonyl group of HX forming imines, or 1,4 addition to the β-carbon of the conjugated double bond of HX forming Michael adducts. Moreover, the Michael adduct can react through the carbonyl group with other primary amino groups of chitosan forming imines and causing a cross-linking effect. Table S1 also represents the swelling values at pH 3 of these runs. It can be observed from Table S1 that a similar DS does not mean a similar release of HX; for example, if runs 11 and 12 are compared, both films were functionalised at 35 °C and employing the highest amount of HX (2 g), but in the film of run 11 a catalyst was not used whereas in the film of run 12, 100 μL of catalyst was used, resulting in very similar DS, 74 ± 3 and 77 ± 9, respectively. However, the release of HX was 38.80 ± 0.70 mg/L_{air}·g_{film} for run 11 and 17.15 ± 1.07 mg/L_{air}·g_{film} for run 12, notably different. Table S1 also shows that the swelling value was higher for the film functionalised without catalyst (run 11). These results mean that although the amount of HX grafted to the film is similar in both runs, the amount of HX anchored to chitosan in the form of conjugated imines is greater when the reaction is carried out without catalyst; whereas in run 12 the formation of Michael adducts is favoured which hinders the release of HX and give rise to lower swelling values which can be due to a cross-linking effect. This behaviour can also be observed when comparing runs 1 and 2 which have similar DS but the swelling and HX release is lower for run 2 where the reaction was carried out at high temperature.

3.1.2. Model validation

According to the experimental design, the optimal conditions to develop CSHX films with maximum aldehyde release were determined to be CS:HX weight ratio of 2:2, the absence of catalyst, and a reaction temperature of 10 °C. These films were then developed and a validation experiment was performed under the optimal conditions owing to verify the accuracy of the model.

The HX release from these films was measured at pH 3, and the experimental result was statistically evaluated with the predicted value using a Student's *t*-test. The experimental HX release at pH 3 was 42.98 ± 4.45 mg/L_{air}·g_{film}, while the theoretical value was 43.18 showing that there was no significant difference between the experimental result and the predicted value.

3.2. Characterisation of optimised CSHX films

3.2.1. Degree of substitution and acid stability of CSHX films

Degree of substitution (DS, %) of CSHX (R_A, R_B and R_C) films was evaluated after film functionalisation and also for films immersed in buffered aqueous solutions at 23 °C for 21 days at pH 3 and pH 7 with the objective of promoting the hydrolysis of the imines, and the results are shown in Table 3. The hypothesis behind this experiment is that if no Michael-adducts and cross-links are formed, DS should be closed to zero

Table 2
ANOVA results for aldehyde release (mg/L_{air}·g_{film}) in the trans-2-hexenal-imine chitosan films.

Factors	Sum of squares	Freedom degree	Mean square	F	*p*-value
A:Temperature	1,175,250	1	1,175,250	72.36	0.0004
B:Catalist	1,143,840	1	1,143,840	70.43	0.0004
C:Trans-2-hexenal	545,757	1	545,757	33.60	0.0022
AA	398,878	1	398,878	24.56	0.0043
AB	248,517	1	248,517	15.30	0.0113
AC	159.012	1	159.012	0.01	0.9250
BB	18,244.7	1	18,244.7	1.12	0.3377
BC	53,622.3	1	53,622.3	3.30	0.1289
CC	10,634.9	1	10,634.9	0.65	0.4552
Error total	81,209.9	5	16,242.0		
R^2	97.7967				

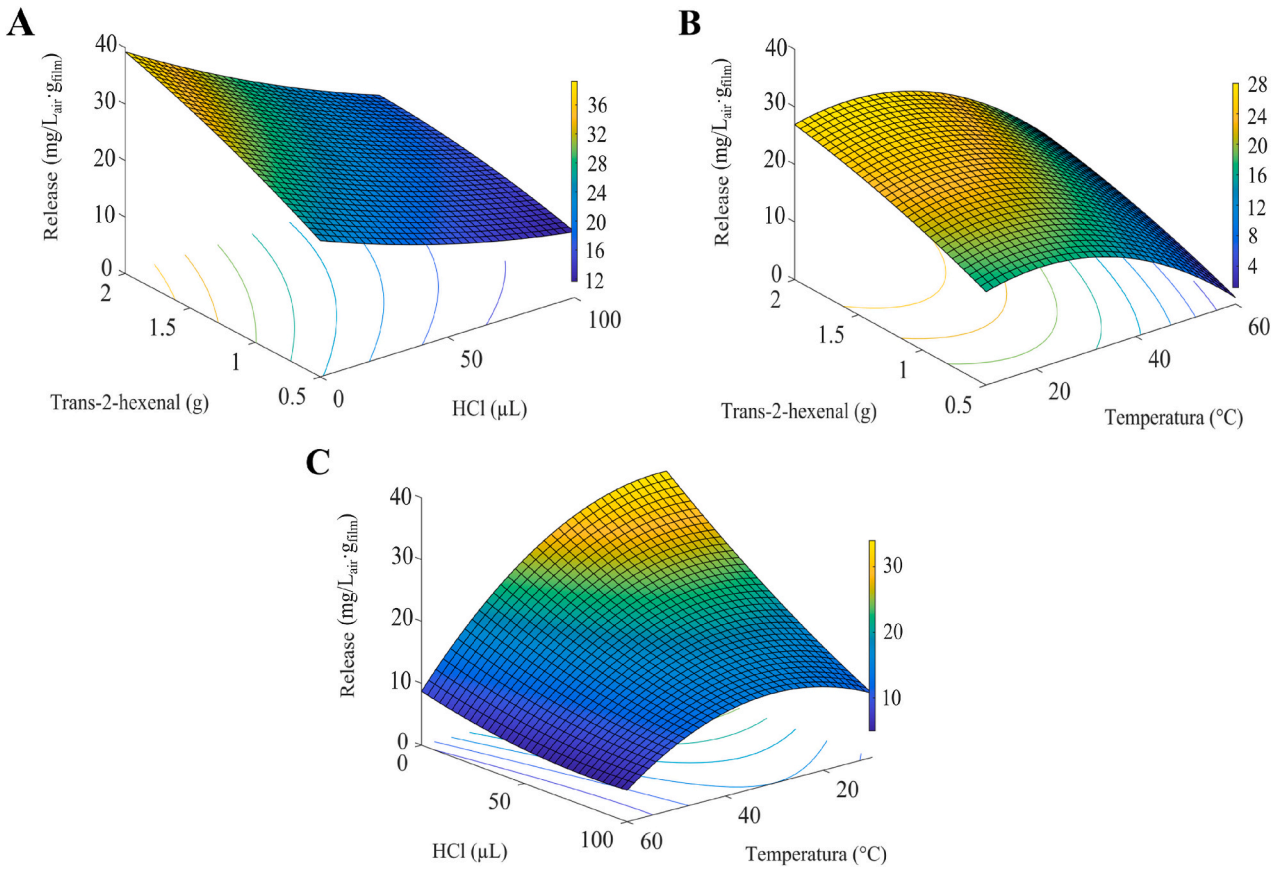

Fig. 3. Release response surface plots for HX films. A) Effect of HX and catalyst (temperature fixed at the centre point) B) Effect of HX and temperature (catalyst fixed at the centre point) C) Effect of catalyst and temperature (HX fixed at the centre point).

Table 3
Degree of substitution (%) of chitosan films synthesised with trans-2-hexenal at 10 °C (CSHX-RA, CSHX-RB, CSHX-RC) before and after exposure to buffered solutions at pH 3 and 7 at 23 °C for 1, 7, 14 and 21 days.

Films	DS (%)	DS $_{pH3}$ (%)				DS $_{pH7}$ (%)			
		Day 1	Day 7	Day 14	Day 21	Day 1	Day 7	Day 14	Day 21
CSHX-R$_A$	74.0 ± 2.0^{cD}	31.0 ± 2.0^{cB}	24.0 ± 2.0^{cA}	22.9 ± 0.3^{cA}	24.0 ± 1.0^{cA}	46.0 ± 2.0^{cC}	32.9 ± 0.2^{cB}	31.6 ± 0.7^{cB}	31.5 ± 0.6^{cB}
CSHX-R$_B$	57.0 ± 1.0^{bE}	23.0 ± 3.0^{bBC}	18.0 ± 2.0^{bAB}	17.0 ± 1.0^{bA}	17.4 ± 4.2^{bA}	38.0 ± 1.0^{bD}	24.6 ± 0.5^{bC}	25.0 ± 2.0^{bC}	23.0 ± 1.0^{bBC}
CSHX-R$_C$	39.5 ± 0.9^{aD}	17.0 ± 2.0^{aB}	11.0 ± 2.0^{aA}	9.3 ± 0.8^{aA}	10.0 ± 2.0^{aA}	25.0 ± 1.0^{aC}	18.0 ± 1.0^{aB}	18.2 ± 0.5^{aB}	16.0 ± 2.0^{aB}

Statistically significant differences ($p \leq 0.05$) are indicated by different superscripts in the same row (A-E) and column (a-c).

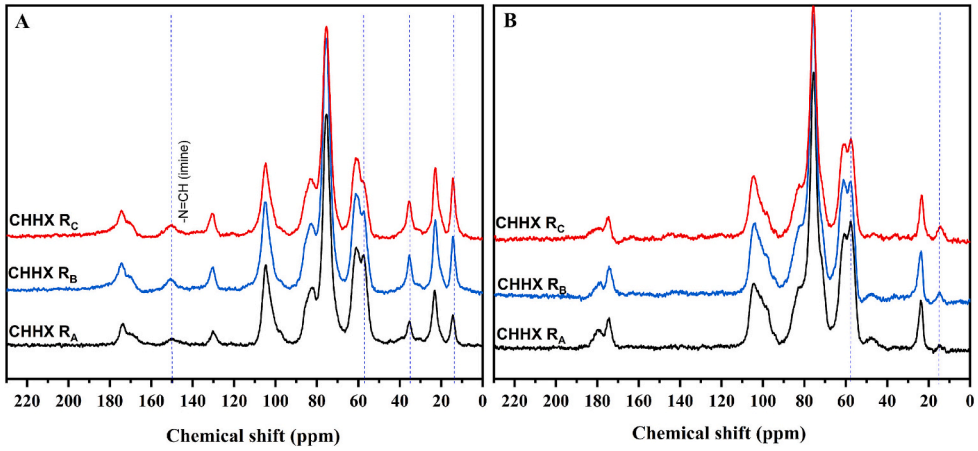

Fig. 4. Solid state ^{13}C NMR of CSHX films (R_A, R_B and R_C) before hydrolysis (A) and after immersion in buffer solution at pH 3 (B). For easier visualization, blue dashed lines indicate the appearance of new peaks assigned to trans-2-hexenal modification.

indicating that imines are hydrolysed, and chitosan films should disintegrate at pH 3.

Initial results before hydrolysis indicated that as CS:HX weight ratio increased, the incorporation of HX in the film increased, resulting in a greater DS. Thus, DS increased from 40 % for the film functionalised employing 2:0.5 ratio (Rc) to 74 % for that using a 2:2 ratio (R_A). In terms of imine stability, as can be seen in Table 3, DS decreased over time due to the hydrolysis of the imine bond and subsequent release of the HX. There was a substantial decline of DS at day 7, and did not change significantly in the sequent weeks. This reduction was more pronounced at pH 3 than at pH 7, due to the susceptibility of imines to hydrolysis in acid conditions. From the results of this experiment, it can be concluded that although functionalised chitosan films are subjected to acid hydrolysis, some residual HX remains covalently anchored to CSHX-R_A, -R_B and -R_C films forming Michael-adducts, which are not hydrolysed; moreover, the remaining HX in the film after hydrolysis increases as the concentration of aldehyde used for film functionalisation increases.

Further assessment of the chemical structure of CSHX (R_A, R_B and R_C) films was performed using solid-state ^{13}C NMR CP-MAS spectroscopy. Resonance peaks corresponding to raw chitosan films have been reported elsewhere [20] at 174 ppm (C=O) from chitosan acetyl group), 105 ppm (C1), 82 ppm (C4), 75 ppm (C5, C3), 61 (C6), 58.0 (C2) and 23 ppm (—CH3—).

The spectra corresponding to the CSHX (R_A, R_B and R_C) before hydrolysis (Fig. 4A) showed a decrease of the intensity corresponding to the resonance peak at 58 ppm, confirming the successful grafting of HX onto the chitosan backbone. In addition, they showed the appearance of resonance bands located at 13, 21 and 34 ppm which can be assigned to the hydrocarbon chain linked to the double bond of trans-2-hexenal. Resonance peaks at 120 and 129 ppm are consistent with carbons corresponding to the imine conjugated double bond, the intensity of the peaks was higher as the amount of HX increased. The imine (C=N) signal was observed at 145 ppm, confirming the formation of imine bonds resulting from the reaction between the aldehyde group of HX and the primary amine group of chitosan. It can be observed that the intensity of the peak corresponding to the imine signal increased and the concentration of HX in the samples increased.

When the CSHX (R_A, R_B and R_C) films were immersed in buffered medium at pH 3 (Fig. 4B), the imine (C=N) signal disappeared which confirmed the hydrolysis of the imine at acid pH thus triggering the HX release. Nonetheless, the presence of the resonance band located at 13 ppm together with the subtle decrease in the intensity corresponding to the resonance peak at 58 ppm ascertained some grafting of HX to the chitosan backbone that could confirm the occurrence of non-hydrolysed Michael-adducts.

3.2.2. *Swelling behaviour*

Water sorption capacity of CSHX-R_A, -R_B and –R_C films was evaluated in buffered solutions at pH 3 and 7 (Fig. 5). As can be seen in Fig. 5C, control chitosan films dissolved after immersion in buffer solution at pH 3, as the pKa of chitosan is around 6.5. However, CSHX films swelled in acid medium, and the swelling increased with time due to imine hydrolysis, moreover, the dimensions of the films also experience a

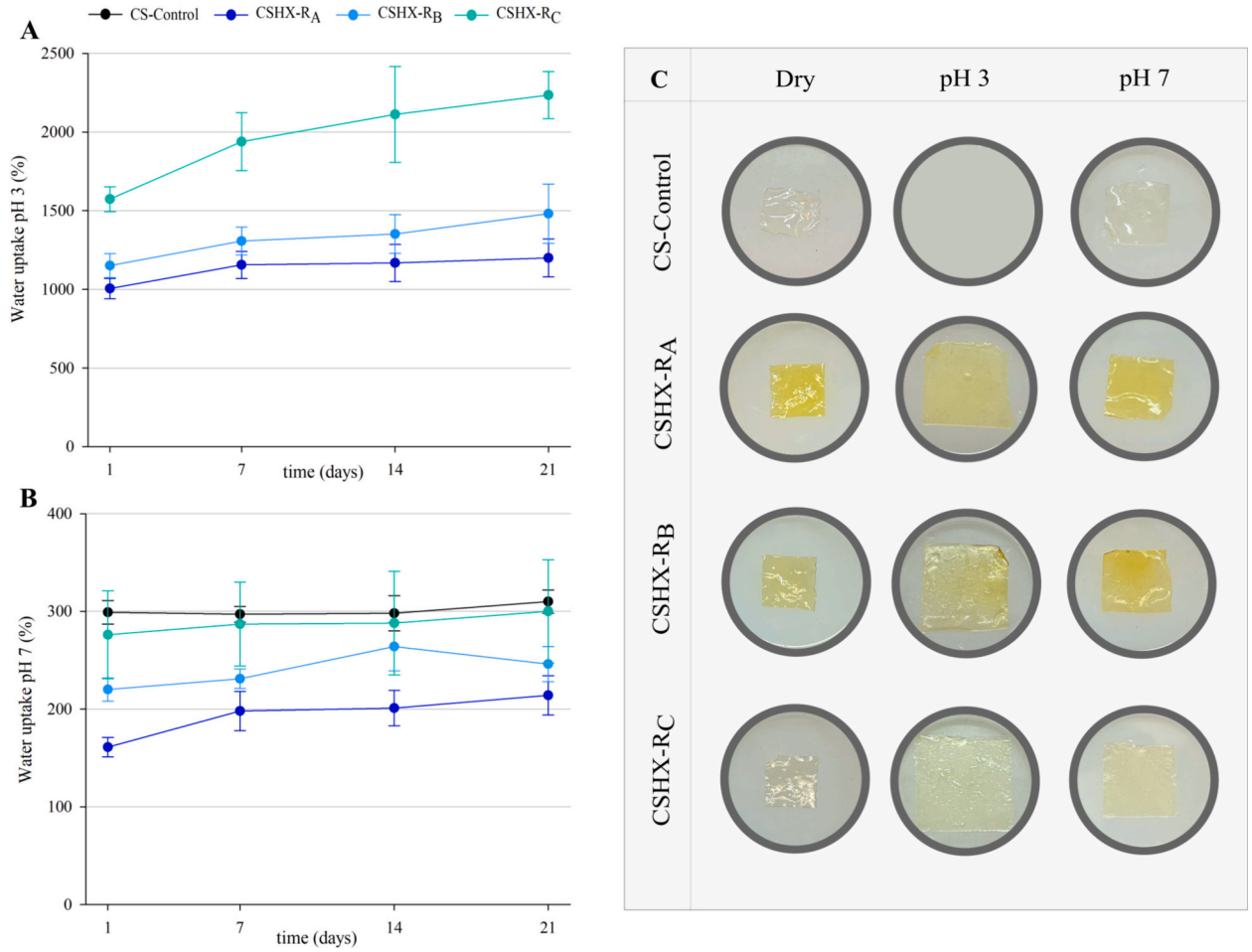

Fig. 5. pH evolution on water uptake (%) of control chitosan film (CS-Control) and films synthesised at 10 °C with different amounts of trans-2-hexenal (CSHX-R_A, CSHX-R_B, CSHX-R_C) immersed in buffered medium at pH 3 (A) and pH 7 (B) at 23 °C for 21 days. Comparative images of the control and CSHX films are presented, illustrating their appearance before and after immersion at pH 3 and pH 7 for 21 days at 23 °C (C).

considerable increased (Fig. 5A); however, the films did not disintegrate after 21 days of immersion in the acid buffer (Fig. 5C). These results are explained by the cross-linking effect of HX through the formation of Michael adducts and imines as is depicted in Fig. 1. Moreover, the swelling decreased when the amount of HX used for the formation of CSHX films increased, indicating the development of a more cross-linked matrix.

Regarding the swelling behaviour of the films at neutral pH, control chitosan films exhibited the highest swelling values at pH 7 due to its high number of free amino groups interacting with water, whereas the swelling in films functionalised with HX was not affected significantly by time and this parameter was greater for films functionalised employing the lower concentration of HX, due to the development of a less cross-linked structure. These results are in agreement with those obtained in Table 3 that show that although functionalised chitosan films are subjected to acid hydrolysis, some residual HX remains covalently anchored to CSHX-R_A, -R_B and -R_C.

3.2.3. Kinetics of trans-2-hexenal release

Gas chromatography in a dynamic system was used to analyse the impact of pH on the imine bond hydrolysis and release of trans-2-hexenal molecules previously grafted onto CSHX films. In this dynamic system, a constant stream of inert gas flowed through the chamber to prevent the build-up of volatile in the headspace and to allow the volatile release from the film until its exhaustion, avoiding

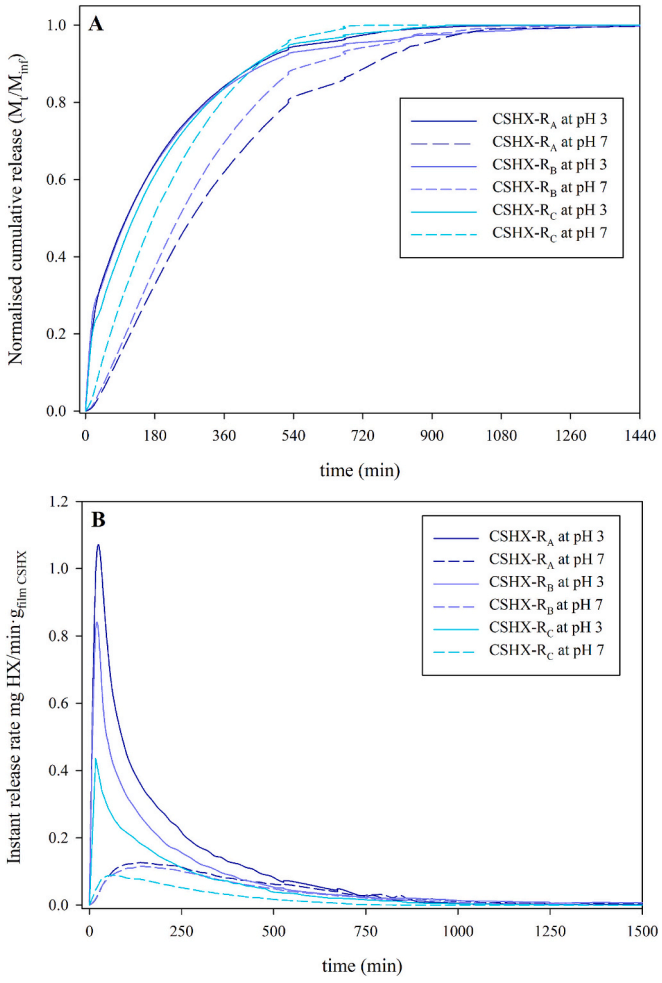

Fig. 6. Trans-2-hexenal release kinetics expressed as normalised cumulative release (M_t/M_{inf}, (A) and instant release rate (mg HX/min·g_{film}) over time from CSHX films exposed at pH 3 and pH 7 and flowing a constant air flow of 12 mL/min at 25 °C during 24 h.

that any partition equilibrium could affect the volatile release kinetics from the film. Fig. 6 shows the release kinetics of trans-2-hexenal from film expressed as normalised cumulative release $M_t/M_{infinite}$ (Fig. 6A) or as instant release rate (mg HX/min·g_{film}) (Fig. 6B), over time for CSHX films at pH 3 and 7. As can be seen in Fig. 6A, the release rate was higher at pH 3 than at pH 7 for all the samples studied. Fig. 6B shows that the maximum amount of HX in the headspace of the studied system was reached after 17 min of running the CSHX films with buffered medium at pH 3 and after 2 h at pH 7.

These results were consistent with those shown in the previous sections, where the hydrolysis of the imine bond was highly affected by the pH. These results were also in accordance with those published by other authors. As an example, Zou et al. [23] grafted polyethyleneimine onto cellulose nanofiber films using Schiff's base and found that the cumulative release rate increased rapidly under acidic conditions, but increased slowly and did not change in a short time under neutral and weakly alkaline conditions. By examining the release rate of the CSHX films in relation to the different HX contents, at pH 3 the HX content has no effect on the rate of imine hydrolysis. However, at pH 7, the HX content of the films significantly affected the release rate. This correlation is related to the swelling behaviour of the film at pH 7 (Fig. 5B). At pH 7 there is a slow hydrolysis of imine, so a higher HX content means fewer amino groups available, resulting in less swelling of the matrix and therefore slower release.

As for the total concentration released per gram of film, it was obtained by calculating the area under the curve and the results are presented in Table 4. The results showed that aldehyde release decreased with decreasing amount of aldehyde used at both pH 3 and pH 7. Specifically, when the amounts of HX were 1 g (R_B) and 0.5 g (R_c), there was a decrease of 30 % and 54 % at pH 3, and 27 % and 59 % at pH 7, respectively, in aldehyde release.

On the other hand, the profile of the HX release plots from films treated at pH 3 (Fig. 6A) resembles that of an exponential tending to a maximum, profile that can correspond to a Fickian desorption. To check this point, experimental data were fitted to the solution to second Fick's law for a plane sheet in which the concentration of the releasing molecule does not affect the process kinetics (Eq. (4)):

$$M_t \Big/ M_{inf} = \left[1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} exp\left(-\frac{D(2n+1)^2 \pi^2 t}{4\ell^2}\right)\right] \quad (4)$$

where ℓ is the thickness of the film and D the diffusion coefficient value. The fitting of this equation to experimental data shown in Fig. 6A was carried out using the Solver tool of Excel Pro (Microsoft Office Professional Plus 2019), solving for D with the objective of minimising the summation of squared differences between experimental and theoretical M_t/M_{inf} values.

CSHX films exposed to pH 3 presented a release profile that is perfectly fitted by Eq. (4). The D values obtained were $(2.4 \pm 0.2)\cdot 10^{-14}$ m^2/s for CSHX-R_A, $(2.3 \pm 0.2)\cdot 10^{-14}$ m^2/s for CSHX-R_B and $(2.2 \pm 0.2)\cdot 10^{-14}$ m^2/s for CSHX0-R_C, with all experimental values matched with relative errors below 10 %. Fig. 7 shows as an example the experimental data and the theoretical values obtained using Eq. (4) for sample CXHX-R_A at pH 3 (Fig. 7A) together with a similar plot for CXHX-R_A at pH 7

Table 4
Total amount of trans-2-hexenal released from CSHX films exposed at pH 3 and pH 7 and flowing a constant air flow of 12 mL/min at 25 °C during 24 h.

Films	HX release (mg HX/g_{film})	
	pH 3	pH 7
CSHX-R_A	170 ± 5^c	68 ± 6^c
CSHX-R_B	119 ± 9^b	50 ± 7^b
CSHX-R_C	79 ± 1^a	27 ± 2^a

Values per column with the same letter within a response show no significant difference ($p > 0.05$).

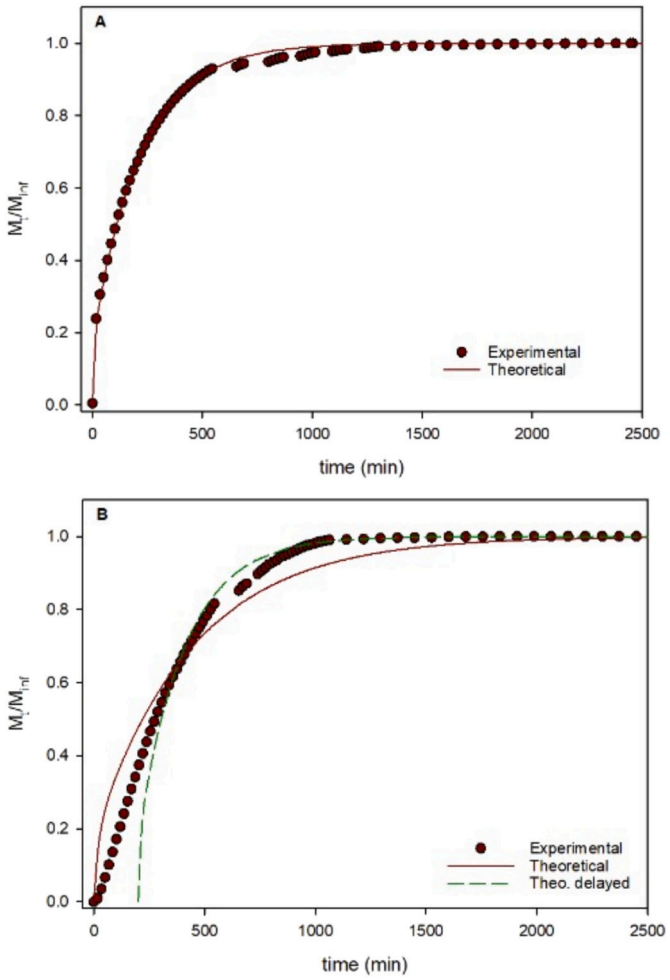

Fig. 7. Release of HX from CXHX-R_A films exposed to pH 3 (A), and pH 7 (B). Dots are experimental data, and curves are theoretical Fickian curves obtained by data fitting to Eq. (4).

(Fig. 7B). As can be seen in Fig. 7A, the fitting is excellent throughout the whole release process, indicating that the HX release is governed by the diffusion of the HX molecules through the biopolymeric matrix, the imine hydrolysis being much faster than the diffusion process. Also, the constant D value obtained for the three films are indicative of the accomplishment of the requirements of the Fick's solution represented in Eq. (4).

CSHX films exposed to pH 7 presented a release profile that cannot be fitted by Eq. 4. The D values obtained by fitting were $(1.1 \pm 0.2)\cdot10^{-14}$ m^2/s for CSHX-R_A, $(1.3 \pm 0.2)\cdot10^{-14}$ m^2/s for CSHX-R_B and $(1.8 \pm 0.2)\cdot10^{-14}$ m^2/s for CSHX-R_C, with all experimental values matched with large relative errors especially at the initial and final parts of the release process. These D values show first that the release is slower than at pH 3, and second, that depend on the concentration of HX, being faster as HX presence decreases. This latter result could be indicative that as HX in the film increases, increases the matrix cross-linking reducing the rate at which the HX molecules are desorbed. Attending to the profile of Fig. 7B, the experimental values appeared to be delayed with respect to what corresponded to a Fickian process. This delayed could be caused by a slow hydrolysis of the imine bond, that retains the HX molecule as to compete with the diffusion, affecting the release process. Fig. 7B shows the experimental data, the curve profile obtained by fitting Eq. (4) with $D = 1.1\cdot10^{-14}$ m^2/s, and a second curve that corresponded to the fitting of the CSHX-R_A exposed to pH 3, that is, with a $D = 2.4\cdot10^{-14}$ m^2/s with 200-min delay, showing that after a short period, the release becomes controlled by a Fickian diffusion. This latter curve appears to confirm that the process is governed by two processes the imine hydrolysis and the diffusion in the matrix.

3.2.4. Antimicrobial activity of CSHX films

The use of trans-2-hexenal (HX) against *Penicillium expansum* and *Escherichia coli* is crucial due to the significant risks these microorganisms pose to food safety and quality. *P. expansum* is one of the world's leading post-harvest pathogens, known to cause blue mould decay in pectin-rich fruits. In addition, this fungus is a producer of harmful mycotoxins, such as patulin, which can pose serious health risks to consumers [24]. Similarly, *E. coli* is a common foodborne pathogen that is frequently associated with outbreaks of contamination in fruit and vegetables [25]. In this context, the antimicrobial properties of trans-2-hexenal have been thoroughly investigated against both bacteria and fungi [19,26]. In this study, and before evaluating the antimicrobial efficacy of optimised films, the capacity of trans-2-hexenal in its vapour phase was tested against both types of microorganisms. The aldehyde showed significant activity against both microorganisms; however, it was more active against the fungus, with MIC and MMC values of 1 μL/plate and 2 μL/plate for *P. expansum*, and 2.5 μL/plate and 7.5 μL/plate for *E. coli*, respectively.

The antimicrobial activity of CSHX-R_A, R_B and R_C films was evaluated using a double-plate system that created a micro-atmosphere containing the antimicrobial volatile release from the film. This evaluation was carried out under conditions where films were exposed to buffer solutions at pH 3 and 7, and using 7.5 mg and 50 mg of film for fungi and bacteria, respectively. The films and culture medium remained physically separated, which ensured that the antifungal and antibacterial properties were attributed only to the aldehyde released within the double-plate system. In particular, no significant differences in microbial growth were found between samples treated with control chitosan films and those without any film. Hence, the data being presented pertains exclusively to the control group that does not include a chitosan film for both microorganisms. Fig. 8 shows the antimicrobial activity of CSHX films against *P. expansum* and *E. coli* when were activated in a buffered solution at both pH 7 and 3, whereas the evolution of fungal inhibition of these films against *P. expansum* during 10 days of incubation at 26 °C is shown in Table S2 of the supplementary material. As can be seen in the Fig. 8, the growth of *E. coli* was not inhibited when CSHX films were immersed in a buffer solution at pH 7, whereas certain inhibition was observed against *P. expansum*, which increased with the content of HX released by the film. However, CSHX films inhibited the growth of both microorganisms when immersed in an acidic aqueous buffer solution (pH 3), demonstrating the strong effect of pH on the hydrolysis of the imine bond and release of the antimicrobial aldehyde. pH-responsive HX-imine-CS films are shown to be more effective to inhibit the in vitro growth of *P. expansum* than other systems used for stabilisation and controlled-release of HX. For example, Wang et al. [26] prepared a starch-based antifungal coating incorporating trans-2-hexenal encapsulated in β-cyclodextrins, but the system did not completely inhibit the in vitro growth of *P. expansum*.

4. Conclusions

In this study, the synthesis of pH-responsive antimicrobial films based on the reversible immobilisation of trans-2-hexenal in chitosan through imine formation was optimised using response surface methodology (RSM) with the objective to avoid the chemical crosslinking effect of the aldehyde, and to maximize the formation of conjugated imines and by hence the release of the volatile aldehyde. The accuracy and reliability of the predictive model generated by RSM were experimentally validated, confirming the effectiveness of the proposed conditions. The optimised films showed excellent antimicrobial properties against bacteria and fungi, indicating their potential as an effective antimicrobial system for the design of antimicrobial active packages. Optimised chitosan films did not dissolve in acidic aqueous solutions in

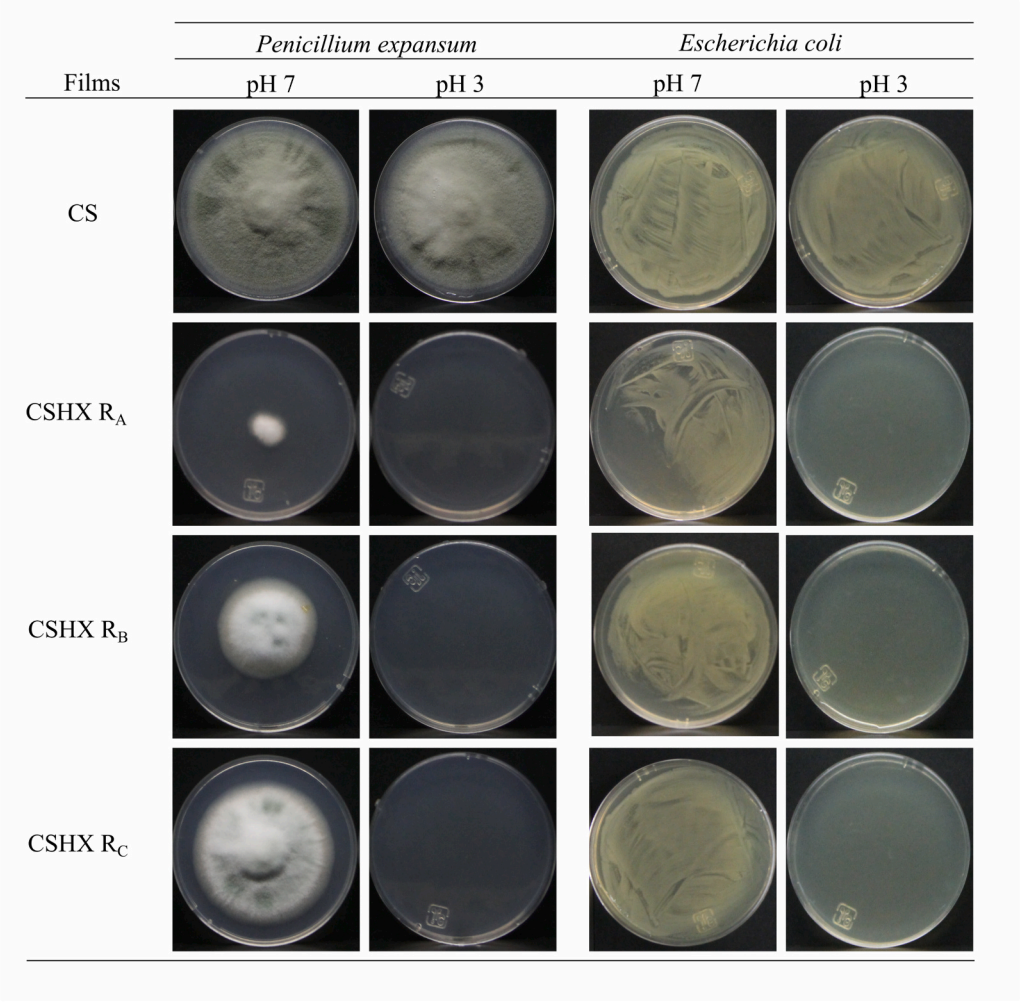

Fig. 8. Antimicrobial activity of CSHX films (7.5 mg) against *P. expansum* after 7 days of incubation at 26 °C, and against *E. coli* (50 mg of film) after 24 h at 37 °C. The images illustrate both the untreated control samples and those treated with CSHX films activated at pH 7 and 3.

contrast to control chitosan film. That behaviour shows that some level of cross-linking could not be completely avoided. This feature may be advantageous in certain applications where film stability is required under acid conditions. Overall, these results highlight the effectiveness of trans-2-hexenal for the formation of acid stable and pH-responsive antimicrobial chitosan films. This research provides valuable insights into the use of naturally occurring α,β-unsaturated aldehydes for the development of pH-responsive antimicrobial films for the design of smart active packaging.

CRediT authorship contribution statement

Patricia Esteve-Redondo: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Raquel Heras-Mozos:** Writing – original draft, Methodology, Investigation. **Rebeca Hernández:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Rafael Gavara:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis. **Pilar Hernández-Muñoz:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.143303.

Data availability

Data will be made available on request.

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