Nutrition and Food Sciences Research Vol 5, No 1, Jan-Mar 2018, pages:23-30

Original Article

Inhibition of Coliform Bacteria in Ultra-Filtrated Cheese Packed in Nanocomposite Films Containing Cloisite30B- Metal Nanoparticles

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Received: September 2017 **Accepted:** November 2017

ABSTRACT

Background and Objectives: Antimicrobial active packaging with metallic nanoparticles is used as antimicrobial agent in the packaging of food. This study considers the coliform retarding ability of antimicrobial packaging in ultra-filtrated (UF) cheese.

Materials and Methods: Plastic films based on low-density polyethylene containing organoclay (cloisite 30B) and different percentages of Ag and CuO nanoparticles produced by extrusion method. Coliform bacteria growth in UF cheese was investigated. The release of nanoparticles from nanocomposite into a food simulant was also assessed.

Results: An amount of 3.17-log cfu/g and 0.75-log cfu/g reduction for coliform bacteria obtained for nanocomposite and control films, respectively after four weeks of storage at 4 °C. Nanocomposite film with 1.3% Ag and 2.7% CuO in LDPE matrix introduced as optimum point by Design Expert 7.1.5 software. Microbial model for decreasing growth of coliform bacteria in this product was also determined. Validation of optimum point was carried out using a one-way ANOVA. It was shown that ther is a non-significant ($p>0.05$) difference with its repeat and a significant ($p<0.05$) difference with the pure film.

Conclusions: Plastic nanocomposite films containing nanoparticle of organoclay and metal can decrease the severity of food processing and application of chemical preservatives in the food industries.

Keywords: Antimicrobial; Nanocomposite; Nanoparticles; Packaging; UF cheese

Introduction

Antimicrobial food packaging has an important role to decrease or inhibit the growth of microorganisms (1). One kind of active packaging is antimicrobial packaging. In this technology, food, headspace and the packaging material interact to gain desired achievements (2, 3).

There is a special interest to clay nanofillers in packaging material (4), because they use in polymer nanocomposites with better polymeric and barrier characteristics, tensile strength, and thermal stability than usual composites (5). Furthermore, clay minerals have antimicrobial property and can absorb antimicrobial agents. Some researchers investigated the microbial capacity of antibacterial compounds, such as cetyl pyridinium, cetyltri methyl ammonium, Ag ions, immobilized on montmorillonite against *Escherichia coli* and *Enterococcus faecium* bacteria (6). Some researchers have considered the details of antibacterial action of clay changed by incorporated copper (7, 8). Beigmohammadi et al. (2016) produced LDPE polymer incorporated with different percentages of Ag-NP, CuO-NP and ZnO-NP by extrusion method for packaging of UF cheese. Results showed metal nanoparticle at a maximum content of 1% could have antibacterial effects on coliform bacteria (9). Peighambardoust et al. (2016)

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produced nano-composite films based on LDPE polymer joined with modified montmorillonite including cloisite 15A, cloisite 20A and cloisite 30B by extrusion method in packaging of UF cheese. They gained the same results with a more than 2-log reduction for coliform load (10).

UF cheese is a very popular cheese group in the Mediterranean region. Different researches have shown the influence of antibacterial compound on coliform bacteria in cheese (11, 12). The antimicrobial activity of polymer/clay nanocomposite films has been considered with different biopolymer based nanocomposite films such as chitosan (13), poly (-lactide) (PLA) (14), whey protein isolate (WPI) (15). To our best knowledge, few researches have been done on real system of foods to test the ability of food packaging material based on a polymeric matrix with different kind of metallic nanoparticle integrated to change organoclay with metal nanoparticles. There is no information about such study on UF cheese. The objective of this research is the utilization of the novel nanocomposite films for cheese packaging composed of LDPE, with different percentages of Ag and CuO nanoparticles joined in cloisite 30B. In this research, UF cheese produced and packed in the nanocomposite films and the coliform load was evaluated weekly. Optimization of the nanocomposite films carried out using D-optimal test in a combined design by Design Expert (version 7.1.5) and microbial model was suggested. Finally, migration test conducted on the nanocomposite with the best antimicrobial properties as the optimum point.

Materials and Methods

Film degree LDPE (LH0075, MFI 0.75 gr/10 min, density 0.921 gr/ml, softening point 94 °C) was optained from Bandar-Imam Petrochemical Co.(Bandar-Imam, Iran) and used for the manufacture of polymer matrix. Nutrino Co. (Tehran, Iran) provided Ag nanoparticles with a medium size of 35 nm and purity of 99.5% and CuO nanoparticles with a medium size of 50 nm and purity of 99%. Cloisite 30B (density 1.98 g/ml, layer space 18.5 °A, moisture<2%) produced by Southern Clay (USA) purcased from Nutrino Co. (Tehran, Iran). PE-g-MA (MFI 1.5-3 g/10 min, melting point 125-130 °C, grafted maleic 0.8-1 %) obtained from Kimya Javid Sepahan Co. (Esfahan, Iran). The white mineral oil-heavy (polyolefin of C17-C30, highly purity, food degree, Unicorn Petroleum Industry, Mumbai, India) kindly obtained from Tabriz Petrochemical Complex (Tabriz, Iran).

To obtain copper exchanged cloisite 30B, five grams of cloisite 30B were distributed in 250 ml of 0.1 mol/L $CuSO_4$. The dispersion holed at 60 °C for 6 h, stirringly. After filtration, the residue cleaned with distilled water to adjust pH value in 5.6, dried at 80 °C overnight (16). Ag exchanged cloisite 30B was obtained similar to the above procedure, 0.02 mol/L $AgNO_3$ solution was used instead of $CuSO_4$ solution. Similar to melt mixing procedure used by other researchers (17-20), a blend of 0.1% (w/w) mineral oil, 4% PE-g-MA, 4% metal nanoparticles-cloisite 30B and 92 % LDPE resins was introduced to a co-rotating twin screw extruder (SMPLATEK, TEK 25 Model, South Korea) with 20 mm of screw diameter. The temperature profile at different area of the twin-screw extruder was 135, 180, 190, 200, 210, 210, 210, and 225 °C. The turn speed of feeder and extruder were 35 and 140 rpm, respectively and pressure of extruder was 12.5 bars that this situation based on LDPE film producer experience of equipment. Furthermore, best mechanical and thermal characteristics decided this circumstance. Because of the included filler (nanoparticle), temperature profile for this nanocomposite is a little different to control LDPE. First, clean pass provided for ingredients by introducing LDPE pure granules, and then LDPE and metallic nanoparticles incorporated into the extruder by feed hopper. Shear and pressure forces through the extruder caused to melt and mix the materials. Molten materials changed to long fiber followed by passing through cold-water basin and forming extruded shape. The arranged granules added into another twin-screw extruder (Castiny Ghioldys, Italy) to manufacture the nanocomposite films with a thickness of 45±5 µm. The heating zone of extrusion process was hold at 185, 215, 218, 223, 223, 239 and 239 °C (9, 10). The produced films were cooled to ambient temperature. The films were chopped in suitable sizes for next application.

The ultra-filtrated cheese manufactured in Manizan Dairy Co. (Kermanshah, Iran) according to method provided by Miocinovic *et al*. (21). The complete milk was pasteurized at a tempreature of 72 °C for 15 sec, concentrated by ultrafiltration system

(APV sw: koch HFK 131, Mesh: 0.01µm, Denmark) 5-fold at 50 °C and 4 bars. The retentate was homogenized at 50 bars and 50-55 °C, and then pasteurized at 78 °C for 15 sec. Retentate injected with starter cultures in the process tank, by Direct Vat Set (DVS) way. Starter cultures included mesophilic cultures of *Streptococcus lactis* subsp. *lactis* and *Streptococcus lactis* subsp. *cremoris* (Mesophile Homofermentative Culture R-708, Item No. 100098 50 U, 50 unit/500 kg, Christian Hansen Co.). The mixture used at a temperature of 30 °C to gain a pH of 4.8-5.0. Finally, salt (3% w/w) and rennet (1g/100 kg) were added into base UF cheese. In order to inhibit the contamination possiblity in production line, the research was carried out in the laboratory and pure coliform added to the cheese base for investigating antimicrobial effect of nanocomposite films (basic microbial load was about 10^7 cfu/g). Various LDPE/ nanocomposite film packs (sealed by heat sealer, Dookht Plast, Iran, with a dimension of 100×120 mm^2) used to package 100 g of UF cheese. For aging, samples were stored at 30 ºC, 5h, and refrigerated at 4±0.5 ºC. The physicochemical characteristics of UF cheese before inoculating by pure culture of coliforms were determined (9, 10).

An amount of 50 g UF cheese sealed in the various nanocomposite films distributed with 450 ml of a sterile Ringer (Merck No. 1.15525.0001, tp: 1035525, Germany) in a stomacher bag and stirred for 1 min. Decimal dilutions of cheese sample were prepared in the same solution for determination of total coliforms using pour plating procedure in violet red bile agar (Merck no. 1.01406.0500, batch vm 629306, Germany), with a top layer of the same medium, incubated at 30 ºC for 24 h (22).

Release of Ag and CuO nanoparticles from LDPE polymer was determined using a food stimulant at 40 ºC for 10 days (23). Migration assay was done on optimum point, nanocomposite film with 1.3 % Ag and 2.7 % CuO. The nanocomposite film with a size of 100×120 mm^2 filled with 200 ml of simulant solution (acetic acid 3%) and the migration results reported to metal migrated/cm^2. Absorbance calculations of copper and Ag nanoparticles performed by electro thermal atomic absorption spectrometry and auto sampler system was employed for building standard solutions of calibration graph from a stock solution with a suitable concentration. Standard solutions of metal ions prepared by diluting stock standard solution (Merck Darmstadt, Germany) with acetic acid 3% (Merck, Darmstadt, Germany) as solvent. Three replications carried out at each measurement and the mean applied for following studies. For determination of nanoparticles, an Analytikjena atomic absorption spectrometer model Nova 400 (Jena, Germany) was used. The circumstances of equipment at determining Cu and Ag nanoparticles were: atomization temperature of 1700 and 1900 ºC, detection limit of 0.13 and 0.05 µg/L, sensibility of 0.23 and 0.15 µg/L. AOAC methods 974.27, 2002b applied for the examination (24).

Statistical Investigation: Data analyzed using Design-Expert 7.1.5 software by applying combined design, D-Optimal procedure for optimization of Ag and CuO nanoparticles in polymer matrix. Mean differences between best point and control sample and repeat of best point that is necessary for validation of results, were determined applying one-way analysis of variance (ANOVA), Tukey HSD and Sheffe tests; ($p<0.05$) by SPSS 17.0 (SPSS, Inc.).

Results

Physicochemical Analysis of UF cheese: Chemical properties of UF cheese was assessed before packaging according to Iranian National standardization Organization (25). However, the objective of this study was not to consider chemical properties of UF cheese packaged in nanocomposite films, this information is for reader about UF cheese.

Antimicrobial test of nanocomposite films: Based on Table 1, all nanocomposite films with cloisite30B- nanometal showed a declined trend for microbial load upon storage. First, the surviving number of coliforms was 6.7×10^5 cfu/g. The cell load of bacteria was fallen to 1.2×10^2–4.4×10^2 cfu/g in various nanocomposite films during 4 weeks, whilst the cell load of control LDPE film only declined to 1.2×10^5 cfu/g. The R-value calculated for all runs and it presented in Table 1.

Table 1. Experimental design, dependent and independent factors for nanocomposite films injected with cloisite30B- metal nanoparticles using combined design test

	Independent Factors			Dependent factors (Response)	
R	Cloisite30B-Ag %	Cloisite30B-	Time/Week	Coliform cfu/gr	R (log)
1	0	4	4	2.6×10^{2}	3.405
2	2	2	1	1.02×10^{5}	0.811
3	0	4	2	1.7×10^{3}	2.589
4	4	0	3	5×10^{4}	1.121
5	4	0	0	6.7×10^{5}	0
6	0	4	4	2.6×10^{2}	3.405
7	2	2	4	3×10^{2}	3.342
8	1	3	0	6.7×10^{5}	0
9	2	2	4	3×10^{2}	3.342
10	2.67	1.33	3	4.7×10^{2}	3.147
11	1.33	2.67	0	6.7×10^{5}	0
12	3	1	1	1.8×10^{4}	1.564
13	4	0	3	5×10^{4}	1.121
14	0	4	1	9×10^{3}	1.865
15	2	2	0	6.7×10^{5}	0
16	0	4	4	2.6×10^{2}	3.405
17	4	0	3	5×10^{4}	1.121
18	4	0	0	6.7×10^{5}	0
19	0	4	0	6.7×10^{5}	0
20	2	2	0	6.7×10^{5}	0
21	1	3	2	1.9×10^{3}	2.541
22	2	2	0	6.7×10^{5}	0
23	2.67	1.33	2	1.7×10^{3}	2.589
24	1.33	2.67	4	4.4×10^{2}	3.176
25	3	1	1	1.8×10^{4}	1.564
26	4	0	4	1.2×10^{2}	3.740

NP means nanoparticle. The LDPE % and PE-g-MA % in all runs are 92% and 4%, respectively.
Rvalue is the difference in bacteria number (log cfu/cm^2) between the non-treated (6.7×10^{5} cfu/g) and treated test samples.

For analysis of response, the suitable model is linear. The suggested model had a significant fit ($p<0.0001$), a non-significant lack of fit, and adequate precision (Adequate precision is a measure of the rang in predicted response relative to its associated error) of 15.5 and explained a high proportion of the total variance, $R^2=0.8339$ (Table 2). Furthermore, interaction nanoparticle-time or antimicrobial effect of Ag and CuO nanoparticle during special time was significant. These conditions are the prerequisite for suggesting a suitable model. Therefore, linear model (Eq. 1) is the suitable model for this design. However, other models for example, quadratic or cubic model are significant too, but interaction nanoparticles - time is not significant.

Eq. 1 shows a description of the effect of independent factors and the influence of binary interaction nanoparticles-time on coliform bacteria number. In considering the influence of each individual nanoparticle in reduction of coliform load, CuO was stronger than Ag. This result provides our objective for declining or deleting Ag-NP because of its price and the toxicity. Investigating nanoparticle-time interaction shows that, negative coefficients represented a declined trend in coliform bacteria and Ag-NP had the most important role. Effects of nanoparticles individually, for two reasons are important. First, they have larger coefficient on the inhibitory influence of coliform load and other, immediate effect of nanoparticle is better for the shelf life of food. Because, during the time the sensory properties of cheese can be influenced. It is clear that Ag-NP in comparison to CuO-NP has more antimicrobial effect, but the objective of our study was to introduce a safe metal nanoparticle with the similar antimicrobial effect of Ag.

Equation (1)

log10 (coliform CFU/gr)=+5.81×Cloisite30B-Ag+5.34×Cloisite 30B- CuO- 0.68×Cloisite 30B- Ag ×Time - 0.81×Cloisite 30B-CuO ×Time

Table 2. ANOVA analysis for selected model by Design Expert software

Source	Sum of Squares	Df	Mean Squares	F-Value	P-value
Model	41.69	3	13.90	36.82	<0.0001 significant
Linear Mixture	3.07	1	3.07	8.12	0.0093
AC	9.40	1	9.40	24.91	<0.0001
BC	16.45	1	16.45	43.59	<0.0001
Residual	8.30	22	0.38		
Lack of Fit	8.30	13	0.64		
Pure Error	0.000	9	0.000		
Cor Total	49.99	25			

Std. Dev.	0.61	R-Squared	0.8339
Mean	4.14	Adj R-Squared	0.8112
C. V %	14.86	Pred R-Squared	0.7646
Press	11.77	Adeq Precision	15.499

A=Ag-NP, B=CuO-NP, C=Time Df=degree of freedom

Based on contour plots of coliform load (Fig. 1), with declining Ag and increasing CuO contents, microbial load is decreasing from the first week to the last week 5.193 log to 2.703 log, respectively. Statistical analyses suggested run 1.3 % Ag-cloisite 30B, 2.7% CuO-cloisite 30B as optimum point, that microbial load of coliforms in this point was reduced 3.176 log cfu/g. Statistical design determines variables for optimization including: Ag-cloisite 30B%, CuO-cloisite 30B%, time, Log10 (coliform) cfu/g and POE (Propagation of Error) (Log10 (coliform) cfu/g, that they have the similar importance, however we changed their importance following based on expectations: Ag-cloisite 30B %: minimize, CuO-cloisite 30B %: in rang, time: equal to 4 weeks, Log10 (coliform) cfu/g: minimize, POE (Log10 (coliform) cfu/g: minimize. Run with 4% Ag and 0% CuO nanoparticles has the most antimicrobial effect (reduction 3.740 log), while we introduce run with 1.3% Ag and 2.7% CuO nanoparticles as optimum point. With using POE, errors decrease.

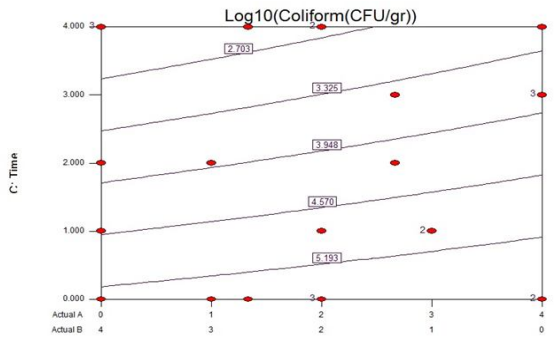


Figure 1. Coliform bacteria Contour plots in UF cheese packaged with nanocomposite films. Red spots on the contour plots shows log10 cfu/gr of coliform in produced nanocomposite films.

Desirability equal to 0.687 is a good condition for the combination of the nanoparticles in LDPE matrix. Furthermore, one of the potentials of Design-Expert software is that it can show optimum point in the form of Ramp chart, too (Fig. 2). Validation of optimum point is illustrated in Table3. To do validating the point, UF cheese packaged in optimum nanocomposite film that suggested by software and assessed for coliform bacteria during 4 weeks, in other word, research repeated only with optimum nanocomposite. To have antimicrobial effect of nanocomposite film, the difference between optimum point and its repeat should not be significant following, the difference of optimum point and control run should be significant. There is not significant difference between optimum point (sample No.1) and its repeat (sample No.2) in Tukey HSD and Sheffe tests ($p<0.05$), while the optimum point has the significant differences with control LDPE (sample No.3). This test done on the cheese sample at last day of storage.

Migration Assay: Because of limited budget, migration test done only on the optimum point. The CuO nanoparticle amount in the food stimulant match with national standard, maximum of 1.5-2 mg/kg in food (26). The level of Ag-NP and CuO-NP released from the nanocomposite film for packaging UF cheese was 0 and 0.1342±0.007 mg/Kg, respectively, which was well below the accepted values. EU safety regulations (EFSA, 2005) mention in particular, the presence of silver ions in food matrices is strongly limited to 50 µg Ag /kg food. The regulation of permitted Ag ion is different. In the US, the FDA agree the use of silver as an antimicrobial agent in bottled water, with a concentration maximum 17 µg Ag /kg (27, 28). Our results for Ag-NP and CuO-NP provide both regulations.

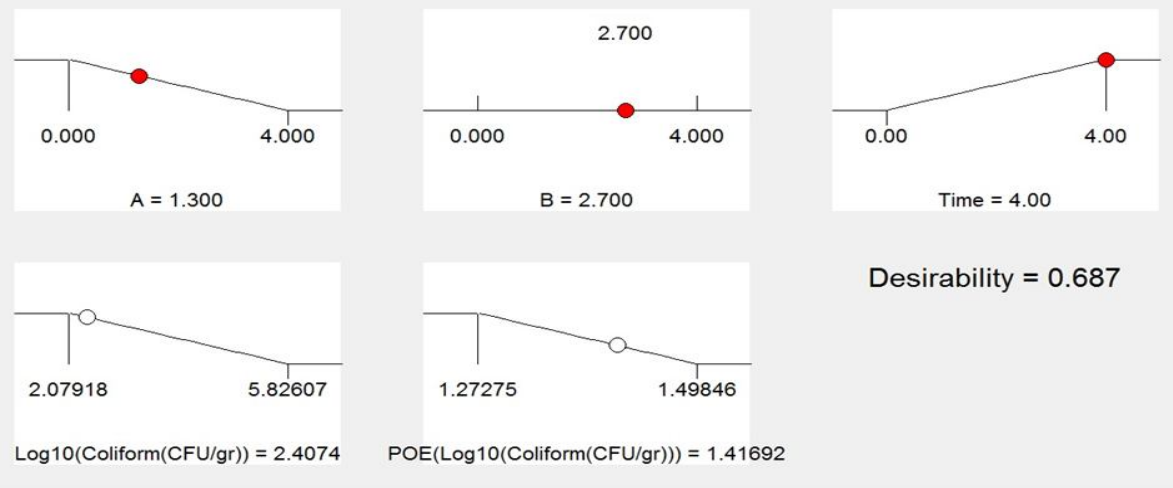


Figure 2. The Ramp chart shows optimum point gained with Design-Expert software.

Table 3. One-way ANOVA analysis for validation of optimum point

	I(X)	(J)X	Mean difference (I-J)	Std.Error	Sig.	% 95 confidence level	
						Lower bound	Upper bound
Tukey HSD	1	2	.00333	.00396	0.693	-0.0088	0.0155
		3	-2.43717*	.00396	.000	-2.4493	-2.4250
	2	1	-.00333	.00396	0.693	-0.0155	0.0088
		3	-2.44050*	.00396	.000	-2.4526	-2.4284
	3	1	2.43717*	.00396	.000	2.4250	2.4493
		2	2.44050*	.00396	.000	2.4284	2.4526
Sheffe	1	2	.00333	.00396	0.715	-0.0094	0.0160
		3	-2.43717*	.00396	.000	-2.4499	-2.4250
	2	1	.00333	.00396	0.715	-0.0160	0.0094
		3	-2.44050*	.00396	.000	-2.4532	-2.4278
	3	1	2.43717*	.00396	.000	2.4245	2.4499
		2	2.44050*	.00396	.000	2.4278	2.4532

1: optimum point 2: validation of optimum point 3: control LDPE
* The mean difference is significant ($p<0.05$)

Discussion

Based on Japanese Industrial standard JIS Z 2801: 2000, from ISO 22196 : 2007 drives, an antimicrobial activity of R>2.0 log cfu/cm^2 is necessary for nano food packaging, to approve antimicrobial potential, as R is the difference in bacterial concentration (expressed in log cfu/cm^2) between the non-treated and treated test samples (19). According to Table 1, some runs had R>2.0 log cfu/cm^2 and were antimicrobial films. The most value for R was 3.74 related to run with only 4% cloisite-Ag that shows Ag nanoparticle is a strong antimicrobial agent against coliform bacteria. With increasing Ag percentage and decreasing CuO percentage in cloisite 30B, R was smaller, though some runs had R less than 2. Another factor is time; it seems nano-clays showed antimicrobial efficiency from second week of storage and continued during storage.

Beigmohammadi et al. (9) and Peighambardoust et al. (10) showed that nanoparticle of Ag, CuO and ZnO maximum 1% and Organoclay including cloisite 15A, cloisite 20A and cloisite 30B maximum 6 % in LDPE polymer had antimicrobial effect on coliform in UF cheese and could reduce coliform bacteria more than 2 log.

Abdolssattari *et al.* (29) packed Lighvan cheese in active LDPE based nanocomposite films containing different metallic and clay nanoparticles and showed that after 28 days of storage, the growth rate of coliform bacteria and *Staphylococcus aureus* significantly ($p<0.05$) decreased as result of application of nanocomposite packaging. Lactic acid

bacteria growth rate was not affected with these nanocomposites.

Peighambardoust *et al*. (30) produced LDPE based polymer incorporating nanoclay modified with copper nanoparticle and found this kind of nanocomposite had antibacterial effect on *Escherichia Coli* and *Staphylococcus aureus* in vitro, however this antimicrobial film was more effective on gram negative than gram-positive. In addition, they obtained maximum 2.2 log reduction for *E. coli* in this nanocomposite with 4% cloisite 30B-CuO.

Malachova *et al*. (31) showed the antibacterial effect of montmorillonite Ag-MMT was more than CuO-MMT on *E. coli*. They showed clays such as montmorillonite could provide an appropriate carrier of metal nanoparticle with antibacterial and antifungal characteristics.

Magana *et al*. (32) considered antimicrobial properties of silver, fixed on montmorillonites in growth inhibition of *E. coli* bacteria. Results showed suitable antibacterial capacity against *E.coli* assessed by disk susceptibility and minimum inhibitory concentration (MIC) method. The results illustrated, the antibacterial activity produced by attached Ag+ on clay, as approved by X-Ray photo electronic spectroscopy (XPS), however the total antibacterial characteristics influenced by ionic silver that is available in contact with the bacteria.

Conclusion

This study showed that application of LDPE nanocomposite films containing metal nanoparticle-cloisite 30B is a new approach for decreasing coliform load in ultra-filtrated cheese. The improved active packaging suggested in our research include 1.3% w/w Ag-NP and 2.7% w/w CuO-Np in LDPE polymer produced by extrusion to packaging UF cheese could be advantageously used to decrease coliform bacteria without toxicity. Nanoparticles of CuO incorporated in cloisite 30B had a higher antimicrobial activity on coliform compared with Ag nanoparticles. This result supplies the objective for decreasing or removing Ag-NP because of its price and toxicity. It is concluded that using antimicrobial films in food packaging systems could decrease or remove the extra amounts of additives and chemical preservatives in food processing as well as a reduction in the severity of processing. Furthermore, combined design method in Design Expert software was a suitable way in designing the experiment, modeling and optimizing the composition of active agents such as nanoparticles of metal in low-density polyethylene matrix.

Acknowledgement

This work was a part of PhD thesis supported by University of Tabriz (Tabriz, Iran). Manizan Dairy Co. (Kermanshah, Iran) is gratefully acknowledged for supplying cheese samples and laboratory facilities in performing this study.

Financial disclosure

The authors declared no financial interest.

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