ANTIMICROBIAL ACTIVITY OF COMPOSITE CHITOSAN BIOFILMS WITH BEESWAX AND CARAWAY ESSENTIAL OIL

ANTIMIKROBNA AKTIVNOST KOMPOZITNOG HITOZANSKOG FILMA SA DODATKOM PČELINJEG VOSKA I ETARSKOG ULJA KIMA

Nevena HROMIŠ, Vera LAZIĆ, Sandra BULUT, Senka POPOVIĆ, Danijela ŠUPUT,
Siniša MARKOV, Natalija DŽINIĆ, Vladimir TOMOVIĆ

University of Novi Sad, Faculty of Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
e-mail: nevena.krkić@uns.ac.rs

ABSTRACT

In this paper, influence of increasing concentrations of beeswax on the antimicrobial activity of chitosan-caraway essential oil biofilm was investigated. Selected microorganisms for the test were *Salmonella Typhimurium* and *Listeria monocytogenes*.

Reduction of *S. Typhimurium* and *L. monocytogenes* viable counts in bacterial suspension in the presence of chitosan films with or without added beeswax amounted over 80 % after 24 h contact time, and over 95 % after 3 h of contact time, respectively and influence of added beeswax on growth reduction was not detected. However, when number of *S. Typhimurium* viable cells was analyzed (log CFU/ml), it was shown that addition of beeswax led to increased activity of films, especially for the films with 36 kg/m³ and 54 kg/m³ of beeswax. When films were transferred, after 24 h in bacterial suspension, onto Petri dishes with nutrient agar and incubated for 24 h on 37 °C, results also showed beeswax contribution to growth inhibition of *S. Typhimurium*.

Key words: chitosan film, essential oil, beeswax, antimicrobial activity, *S. Typhimurium*, *L. monocytogenes*

REZIME

Antimirobno pakovanje hrane, kao jedna od osnovnih aplikacija sistema aktivnog pakovanja, treba da ima mogućnost da onemogući ili inhibira rast mikroorganizama koji izazivaju kvar hrane, kao i patogenih mikroorganizama i, na ovaj način, osigura bezbednost, održi kvalitet i produži održivost hrane.

U ovom radu, ispitan je uticaj dodatka rastuće koncentracije pčelinjeg voska na antimikrobnu aktivnost kompozitnog filma na bazi hitozana sa dodatkom etarskog ulja kima. Kao test mikroorganizmi odabrani su *Salmonella Typhimurium* i *Listeria monocytogenes*.

Aktivnost je određena prema ASTM E2149 metodi, koja predstavlja kvantitativnu metodu određivanja antimikrobne aktivnosti koja se izvodi pod dinamičkim uslovima kontakta. Redukcija vijabilnih ćelija u suspenzijama *S. Typhimurium* i *L. monocytogenes* u prisustvu hitozanskog filma sa etarskim uljem kima, kao i u prisustvu filmova sa pčelinjim voskom iznosila je preko 80% nakon 24h kontakta, odnosno 95% nakon 3h kontakta i uticaj dodatka pčelinjeg voska na redukciju nije bio uočljiv. Sa druge strane, ukoliko se analizira broj vijabilnih ćelija (log CFU/ml), a ne redukcija vijabilnih ćelija (%), može se videti da je dodatak pčelinjeg voska ipak doveo do povećanja aktivnosti filma, posebno u slučajevima sa dodatkom 36 kg/m³ i 54 kg/m³ voska, gde je inhibicija rasta *S. Typhimurium* iznosila 1.9 log jedinica i 1,6 log jedinica, dok je za film bez dodatog voska ova redukcija iznosila 0,7 log jedinica. Kada su filmovi premešteni, nakon 24 h u bakterijskoj suspenziji, na Petri ploče sa nutrient agarom i inkubirani tokom 24 h na 37°C, rezultati su takođe pokazali doprinos pčelinjeg voska inhibiciji rasta *S. Typhimurium*.

Ključne reči: hitozanski film, etarsko ulje, pčelinji vosak, antimikrobna aktivnost, *S. Typhimurium*, *L. Monocytogenes*.

INTRODUCTION

Growing consumer demand for food without chemical preservatives, directed great amount of research towards finding natural alternative substances that could be used for food preservation (Popović *et al.*, 2015). Natural biopolymer chitin and its derivatives showed pronounced antimicrobial activity followed with other positive properties, which all led to extensive research in widening these biopolymers field of application (Chi, 2004; Hromiš *et al.*, 2014).

Chitosan in solid form, in the form of fibers, films, membranes, hydrogels, microspheres and nanoparticles comes only in the surface contact with substrate and its antimicrobial activity depends on surface properties like hydrophilicity, zeta potential and morphology: particle size and shape, membrane thickness (Kong *et al.*, 2010; Kong *et al.*, 2008; Sadeghi *et al.*, 2008; Takahashia *et al.*, 2008). In order to intensify antimicrobial activity of chitosan films, different antimicrobial compounds are being added to chitosan film. This addition is important because film can manifest its antimicrobial activity

only in direct contact with a substrate surface. In order to achieve antimicrobial activity that protects mass of the substrate it is necessary to have antimicrobial compound able to migrate into the substrate. Accordingly, low molecular weight active compounds are added into the chitosan matrix, being subsequently released to migrate into the substrate (Altioek *et al.*, 2010; Coma *et al.*, 2002; Pranoto *et al.*, 2005). Plant essential oils and extracts can be considered as natural alternative in food protection and their use is in accordance with consumer tendency towards natural, minimally chemically treated food (Burt, 2004; Kocić-Tanackov *et al.*, 2013). Two basic limitations in essential oil usage in food industry are pronounced organoleptic properties and loss of activity due to interactions with food components (Gutierrez *et al.*, 2008; Rojas-Graü *et al.*, 2009).

Caraway essential oil and its basic compounds, carvone and limonene showed antimicrobial activity towards different moulds and bacteria (Aggarwal *et al.*, 2002; Dimić *et al.*, 2009; Simić *et al.*, 2008). Literature data about caraway essential oil antimicrobial activity vary, from pronounced activity over

moderate to literature data of lack of activity towards most Gram positive and Gram negative bacteria (Alboofetileh et al., 2014; Cvijović et al., 2010; Mohamed et al., 2013).

In paper which analyzed methanol and ethanol extract of beeswax influence on the growth of five pathogenic bacterial species, three species of microscopic fungi and seven species of yeasts, activity of medium intensity was observed (Kacániová et al., 2012). In paper where effect of coating based on chitosan, beeswax and lime essential oil on *E. coli* DH5a and *R. stolonifer* „in vitro“ and „in vivo“ was analysed, it was shown that in both experiments, „in vitro“ and „in vivo“, coating based on chitosan and beeswax totally inhibited bacterial growth (Ramos-García et al., 2012). Also, Al-Waili (2005) showed moderate growth of *S. aureus* on the nutrient agar with certain content of beeswax.

In this paper, influence of increasing concentrations of beeswax on the antimicrobial activity of composite biofilm based on chitosan with added caraway essential oil was investigated. Selected microorganisms for the test were two pathogenic bacteria, *Salmonella* Typhimurium as Gram-negative and *Listeria monocytogenes* as Gram-positive bacteria.

MATERIAL AND METHOD

Material

Commercial chitosan powder from crab shells, highly viscous (deacetylation degree approximately 80 %) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). Caraway essential oil was purchased from the manufacturer: Herba doo (Belgrade, Serbia) and beeswax was obtained from a local beekeeper. Other used chemicals were of analytical grade.

Film preparation

Chitosan film forming solution was prepared by dissolving chitosan powder in acetic acid (1 % volume concentration) to reach chitosan mass per volume ratio of 10 kg/m³. Solution was left stirring over night on a magnetic stirrer to dissolve chitosan. Caraway essential oil in volume concentration of 1 % and wetting agent Tween 20 (50 % of essential oil volume) were then added, solution was heated in a water bath to 60 °C and stirred with a laboratory stirrer to obtain white homogenous emulsion. In the same water bath, beeswax was melted in 30 mL of deionized water heated to 60 °C. Hot film forming emulsion was added to molten wax and six new different film forming emulsions with beeswax mass per volume ratio of: 0, 18, 36, 54, 72 and 90 kg/m³ were obtained. Emulsions were casted on Petri dishes covered with teflon coating and left to air dry (23±2 °C, 50 ±10 % RH) on a horizontal table surface. Films were labeled according to beeswax mass per volume ratio, as 0, 18, 36, 54, 72 and 90, while 0 (C) was label used for the film without beeswax or caraway essential oil (Table 1).

Table 1. Composition of films

Film label	Chitosan mass per volume ratio (kg/m ³)	Caraway essential oil (% vol)	Beeswax mass per volume ratio (kg/m ³)
0 (C)	10	0	0
0	10	1	0
18	10	1	18
36	10	1	36
54	10	1	54
72	10	1	72
90	10	1	90

Antimicrobial activity

Activity was tested according to the ASTM E2149 (2001) method, which is a quantitative antimicrobial test method, performed under dynamic contact conditions. Gram-positive (*Listeria monocytogenes*) and Gram-negative bacteria (*Salmonella* Typhimurium) were used as test organisms. Fresh inoculants for antibacterial assessment were prepared on nutrient agar (Merck, Germany) at 37 °C for 24 h. The incubated test culture was diluted using a sterilized 3 mM phosphate buffer (KH₂PO₄; pH 6.8) to give a final concentration of 1.5-3×10⁵ colony forming units (CFU)/mL. This solution was used as a working bacterial dilution.

Before analyzing antimicrobial activity of films, cell viability of test organisms was determined in set experimental conditions. Cell viability determination in phosphate buffer showed no significant effect on *S. Typhimurium* cells viability, because there was no decrease or increase in viable cell number during 24 h. However, considerable decrease in *L.monocytogenes* cell number was recorded after 24 h. This is why film antibacterial effect against *L.monocytogenes* was tested only after 3 h (Fig. 1).

Fig. 1. Cell viability of test organisms in phosphate buffer after 3 h and 24 h

Film samples were treated with UV lamp (Kruss, Germany): 254 nm, ½ h for one side of film, cut into test pieces, which had contact surface area of 58 cm² and transferred to a 250 mL Erlenmeyer flask containing 50 mL of the working bacterial dilution. All flasks were capped loosely and shaken for 24 h at room temperature and 120 rpm using a Wrist Action incubator shaker. At different contact periods: 3 h and 24 h, 1 mL of the bacterial dilution was withdrawn, serial diluted and plated in Trypton soya agar (Merck, Germany). The inoculated plates were incubated at 37 °C for 24 h, and colonies were counted. The average values of the duplicates were converted to CFU/mL in the flasks by multiplying with the dilution factor. The antimicrobial activity was expressed as R % reduction of the organism after contact with the test specimen compared to the number of microorganisms cells surviving after contact with the control (Eq.1):

$$\text{Reduction, \% (CFU/mL)} = \frac{(B-A)}{B} \times 100$$

where A represents CFU per mL for the flask containing the treated substrate after the specified contact time and B represents “0” contact time CFU per mL for the flask used to determine “A” before the addition of the treated substrate. Reduction (%) was calculated using average of 4 replicates for A and B.

After 24 h in bacterial solution, in additional test, films were transferred from bacterial suspension onto Petri dishes with

nutrient agar and incubated for 24 h on 37 °C to obtain further insight in antimicrobial activity of the films.

Statistical analysis

Statistical analysis was carried out using OriginPro 8 (OriginLab Corporation, Northampton, MA, USA). All data were presented as mean value with their standard deviation indicated (mean $\pm$ SD). Variance analysis (ANOVA) was performed, with a confidence interval of 95 % ($p < 0.05$). Means were compared by the Tukey test.

RESULTS AND DISCUSSION

Reduction of *S. Typhimurium* viable counts in bacterial suspension in the presence of chitosan film, chitosan film with caraway essential oil, as well as in the presence of films with added beeswax is shown in Fig. 2. For chitosan film without added caraway essential oil and beeswax, 0(C), bacterial growth reduction of 26.96 % (after 3 h contact) and 63.97 % (after 24 h contact) was observed. Considering that in this experiment we tested chitosan in solid form of the film, only surface of the film could come into contact with bacterial suspension (Kong et al., 2010; Kong et al., 2008; Lagaron et al., 2007; Sadeghi et al., 2008; Takahashia et al., 2008). In addition, test was performed in neutral pH 6.8, where ratio of charged amino groups on C-2 chitosan atom is low. This could be one of the reasons for lower antimicrobial activity (Kong et al., 2010). When activity of chitosan film with caraway essential oil against *S. Typhimurium* growth is analyzed, it can be seen that activity increases as the contact time increases, reaching about 80 % after 24 h. When beeswax was added to the film, no significant change could be observed in the film activity ($P > 0.05$), although for films with beeswax concentrations 36, 54 and 72 kg/m³ tested antibacterial activity reached and exceeded 90 %. For the film with highest amount of beeswax low activity was detected. It is possible that high amount of beeswax interacted with other lipophilic compound, caraway oil, and as a result of interaction oil release from the film was slowed or blocked.

Fig. 2. Reduction (%) of viable cell number after 3 h and 24 h of film contact time in *S. Typhimurium* bacterial suspension

If inhibition of *S. Typhimurium* growth in the presence of tested composite films is presented as number of viable cells (log CFU/ml) after selected contact periods of 3 h and 24 h (Fig 3), instead of reduction (%) (Fig. 2), some differences between films with and without beeswax can be observed, especially after longer contact period. Similar to the results for reduction,

chitosan film without added beeswax or caraway essential oil, 0 (C) did not show decrease in viable cell number. After 24 h contact with chitosan film with caraway essential oil, but without beeswax, CFU/ml decreased for about 0.7 log units. Addition of beeswax led to increased activity of films, especially for the films with 36, 54 and 72 kg/m³ of beeswax, where inhibition was 1.9 log cycle, 1.3 log cycle and 1.6 log cycle.

Fig. 3. Number of viable cells, log CFU/ml after after 3 h and 24 h of film contact time in *S. Typhimurium* bacterial suspension

After 24 h in bacterial suspension, films were transferred onto Petri dishes with nutrient agar and incubated for 24 h on 37 °C. Results are presented in Fig. 4. For the film 0 (C), bacterial growth was observed both in contact with the film and in film surrounding. For all the films with added beeswax, bacterial growth was observed in surrounding of films, but there was no growth observed in direct contact with films. Effect of increasing concentration of beeswax on the growth inhibition of *S. Typhimurium* was not clearly noticeable. Lack of bacterial growth in direct contact with films with added beeswax may be due to bacteriostatic effect of films. On the other hand, mechanism of activity of film without beeswax is not clearly bactericidal or bacteriostatic, due to observed growth of bacteria in contact with the film, as well as in the film surrounding.

Fig. 4. Images of *S. Typhimurium* bacterial growth on nutrient agar after film transfer from bacterial suspension

Due to reduction in cell viability of *L. monocytogenes* in experimental conditions after 24 h, only results after 3 h of contact period with films are presented in Fig. 6. It can be

observed from Fig. 5. that mean value of reduction of *L. monocytogenes* in presence of the film 0 (C) was around 40 %. In contact with chitosan film with added caraway essential oil, reduction was above 90 % and similar reduction ($P>0.05$) was observed in all films with added beeswax, except for the film with highest concentration of beeswax, 90 kg/m³, similar to the results obtained for *S.Typhimurium*.

Fig. 5. Reduction (%) of viable cell number after 3 h of film contact time in *L.monocytogenes* bacterial suspension

Inhibition of *L.monocytogenes* in the presence of tested composite films is presented in Fig. 6, as number of viable cells (log CFU/ml) after contact period of 3 h. No contribution of added beeswax to film activity against *L.monocytogenes* was observed, but test could not be performed after 24 h of contact time, where possibly different results might be obtained, as in the case of *S.Typhimurium*.

Fig. 6. Number of viable cells, log CFU/ml after 3 h of film contact time in *L.monocytogenes* bacterial suspension

CONCLUSION

Chitosan-caraway essential oil film resulted in over 80 % inhibition of *S.Typhimurium* after 24 h contact period and over 90 % of inhibition of *L. monocytogenes* after 3 h of contact period, compared to no or 20-30 % reduction of bacterial growth by the chitosan film. However, contribution of beeswax to antimicrobial activity of composite film was also detected after longer contact period in *S.Typhimurium* suspension (1 log cycle lower CFU/ml after 24 h of contact period). Moreover, for the

film without beeswax, bacterial growth was observed in contact with the film, as well as in the film surrounding after transferring film from bacterial suspension into nutrient agar and incubation, while for films with added beeswax, absence of bacterial growth in direct contact with films was detected. Due to reduction in cell viability of *L. monocytogenes* in experimental conditions after 24 h, only results after 3 h of contact period could be tested and in these conditions no contribution of beeswax to antilisterial activity of films could be detected.

ACKNOWLEDGMENT: This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project No. TR31032: Development of production technology of traditional fermented dry sausages with geographical origin to obtain safe products of standard quality (2011-).

REFERENCES

- Aggarwal, K.K., Khanuja, S.P.S., Ahmad, A., Kumar, T.R.S., Gupta, V.K., Kumar, S. (2002). Antimicrobial activity profiles of the two enantiomers of limonene and carvone isolated from the oils of *Mentha spicata* and *Anethum sowa*. *Flavour Fragrance Journal*, 17 (1), 59–63.
- Alboofetileh, M., Rezaei, M., Hosseini, H., Abdollahi, M. (2014). Antimicrobial activity of alginate/clay nanocomposite films enriched with essential oils against three common foodborne pathogens. *Food Control*, 36, 1-7
- Altioek, D., Altioek, E., Tihminlioglu, F. (2010). Physical, antibacterial and antioxidant properties of chitosan films incorporated with thyme oil for potential wound healing applications. *The Journal of Materials Science: Materials in Medicine*, 21, 2227–2236.
- Al-Waili, N.S. (2005). Mixture of Honey, Beeswax and Olive Oil Inhibits Growth of *Staphylococcus aureus* and *Candida albicans*. *Archives of Medical Research*, 36, 10-13.
- ASTM standard E2149-01 (2001). Standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions. ASTM international, Pennsylvania, USA.
- Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, 94 (3), 223–253.
- Chi, S. (2004). Development and characterization of antimicrobial food coatings based on chitosan and essential oils. M Sc Thesis. The University of Tennessee, Knoxville, Tennessee, USA.
- Coma, V., Martial-Gros, A., Garreau, S., Copinet, A., Salin, F., Deschamps, A. (2002). Edible Antimicrobial Films Based on Chitosan Matrix. *Journal Of Food Science*, 67, 1162-1169.
- Cvijovic, M., Djukic, D., Mandic, L., Acamovic-Djokovic, G., Pesakovic, M. (2010). Composition and antimicrobial activity of essential oils of some medicinal and spice plants. *Chemistry of Natural Compounds*, 46 (3), 481-483.
- Dimić, G., Kocić-Tanackov, Sunčica, Pejin, D., Pejin, Jelena, Tanackov, I., Tuco, D. (2009). Antimicrobial activity of caraway, garlic and oregano extracts against filamentous moulds. *Acta Periodica Technologica*, 40, 9-16.
- Gutierrez, J., Barry-Ryan, C., Bourke, P. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*, 124 (1), 91-97.
- Hromiš, N., Lazić, V., Popović, S., Šuput, D., Džinić, N. (2014). Chitosan film with addition of beeswax and caraway essential oil. *Journal on Processing and Energy in Agriculture* 18 (5), 220-224.

- Kacániová, M., Vuković, N., Chlebo, R. Haščík, P., Rovná, K., Cubon, J., Džugan, M., Pasternakiewicz, A. (2012). The antimicrobial activity of honey, bee pollen loads and beeswax from Slovakia. *The Archives of Biological Sciences*, 64, 927-934.
- Kocić-Tanackov, Sunčica, Dimić, G., Mojović, Ljiljana, Pejin, Jelena, Gvozdanović-Varga, J. (2013). Inhibitory activity of the onion essential oil on growth *Cladosporium cladosporioides*, *Emericella nidulans* and *Eurotium spp.* *Journal on Processing and Energy in Agriculture*, 17 (1), 12-16.
- Kong, M., Chen, X.G., Liu, C.S., Liu, C.G., Meng, X.H., Yu, L.J. (2008). Antibacterial mechanism of chitosan microspheres in a solid dispersing system against *E. coli*. *Colloids and Surfaces B: Biointerfaces*, 65, 197-202.
- Kong, M., Chen, X.G., Xing, K., Park, H.J. (2010). Antimicrobial properties of chitosan and mode of action: A state of the art review. *International Journal of Food Microbiology*, 144, 51-63.
- Lagaron, J.M., Fernandez-Saiz, P., Ocio, M.J. (2007). Using ATR-FTIR Spectroscopy To Design Active Antimicrobial Food Packaging Structures Based on High Molecular Weight Chitosan Polysaccharide. *Journal of Agricultural and Food Chemistry*, 55(7), 2554-2562.
- Mohamed, S., Zaky, W., Kassem, J., Abbas, H., Salem, M.M.E., Said-Al Ahl, H.A.H. (2013). Impact of Antimicrobial Properties of Some Essential Oils on Cheese Yoghurt Quality. *World Applied Sciences Journal*, 27 (4), 497-507.
- OriginPro 8, 8.0891 (2008). OriginLab Corporation, Northampton, Massachusetts, USA.
- Popović, S., Lazić, V., Hromiš, N., Šuput, D., Bulut, S., Popović, Lj., Vaštag, Ž. (2015). Influence of guar-xanthan addition on mechanical properties of pumpkin oil cake biopolymer films. *Journal on Processing and Energy in Agriculture*, 19 (4), 179-182.
- Pranoto, Y. Rakshit, S.K., Salokhe, V.M. (2005). Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin. *LWT - Food Science and Technology*, 38, 859-865.
- Ramos-García, M., Bosquez-Molina E., Hernández-Romano J., Zavala-Padilla G. D., Terrés-Rojas E., Alia-Tejagal I., Barrera-Necha L., Hernández-López M., Bautista-Baños, S. (2012). Use of chitosan-based edible coatings in combination with other natural compounds, to control *Rhizopus stolonifer* and *Escherichia coli* DH5a in fresh tomatoes. *Crop Protection*, 38, 1-6.
- Rojas-Graü, M.A., Soliva-Fortuny, R., Martín-Belloso, O. (2009). Edible coatings to incorporate active ingredients to freshcut fruits - a review. *Trends in Food Science & Technology*, 20 (10), 438-447.
- Sadeghi, A.M.M., Dorkoosh, F.A., Avadi, M.R., Saadat, P., Rafiee-Tehrani, M., Junginger, H.E. (2008). Preparation, characterization and antibacterial activities of chitosan, N-trimethyl chitosan (TMC) and N-diethylmethyl chitosan (DEMC) nanoparticles loaded with insulin using both the ionotropic gelation and polyelectrolyte complexation methods. *International Journal of Pharmaceutics*, 355, 299-306.
- Simić, A., Rančić, A., Sokovic, M.-D., Ristic, M., Grujic-Jovanovic, S., Vukojevic, J., Marin, P.-D. (2008). Essential oil composition of *Cymbopogon winterianus* and *Carum carvi* and their antimicrobial activities. *Pharmaceutical Biology*, 46 (6), 437-441.
- Takahashia, T., Imaia, M., Suzukia, I., Sawai, J. (2008). Growth inhibitory effect on bacteria of chitosan membranes regulated by the deacetylation degree. *Biochemical Engineering Journal*, 40, 485-491.

Received: 17. 02. 2017.

Accepted: 11. 04. 2017.