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Conversion of Waste Parasitic Insect (*Hylobius abietis* L.) into Antioxidative, Antimicrobial and Biodegradable Films

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Abstract: *Hylobius abietis* is a plant parasitic insect belonging to the order Coleoptera and which causes severe damages to coniferous forests in Northern and Eastern Europe. This current study is aimed to provide a new viewpoint into the waste of this insect by producing chitosan. Dry insect corpses consisted of 27.9% chitin and 86.2% of the chitin was converted into the chitosan. FT-IR spectra analyses confirmed the purity and the deacetylation degree of the produced chitosan (molecular weight of chitosan; 7.3 kDa). This chitosan exhibited antimicrobial activity against 18 bacterial strains. Further, biodegradable chitosan composite films with β-carotene were produced. Antioxidant activity of chitosan films were found to be higher than chitosan gels; and β-carotene incorporation further increased the antioxidative properties of the chitosan films. This study demonstrated that the waste of parasitic insect like *H. abietis* can be evaluated as a source for production of biodegradable and edible chitosan-based films for applications in food coating.

Keywords: *Hylobius abietis*; biowaste; chitin; chitosan; antioxidant

1 Introduction

Hylobius abietis, known as pine weevil, is a coleopteran species and it is widely spread throughout Europe, Asia and New Zealand. It feeds on the bark of young trees and it is regarded as a major pest of the conifers in Northern and Eastern Europe [1-3]. Adult females lay their eggs under the bark of conifer and the larvae feed on the stem bark of young conifer seedlings [4]. The pest is responsible for growth losses and high mortality (60-80%) in *Pinus sylvestris* L. and *Picea abies* (L.) species [1]. The mortality rate can reach 100% among young conifer seedlings in clear-felling and replanting areas [5]. Especially in the following years of replanting, a large number of the adults and larvae attacked the bark of the seedlings [3]. In current study in Lithuania, where we collected *H. abietis* samples, damage by *H. abietis* is widespread and this aggressive species causes substantial mortality among *Pinus* species [6]. Insecticides are used for the seedling protection, pest corpses are incinerated or buried [3,7]. In our opinion the use incineration procedure as a *H. abietis* control measure wastes a potentially valuable chitin source. This study emphasizes the use of *H. abietis* corpses as a source of chitin and chitosan.

Chitosan is a functional versatile biopolymer, obtained through deacetylation of chitin. Unlike chitin, aminofree group makes chitosan cationic and more suitable for modifications due to its water-soluble nature [8]. Nowadays chitosan has common applications in food / feeding industry, agriculture,

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environmental protection, biotechnology, cosmetics, textile and pharmacology [9]. Also, chitosan is biodegradable, biocompatible and nontoxic and therefore it is used as a biomaterial in biomedical applications [10]. Chitosan is commercially produced from the sea animal waste like shrimp and crab shells. However, there are many other organisms that are widespread and capable of reproducing in large numbers and have chitinous exoskeleton. That sort of organisms can be evaluated for commercial production of chitosan.

It is well known that antioxidant packaging increases the shelf life of food products by preventing the oxidation [11]. Because of its useful and economical property, antioxidant packaging can be indispensable for edible food coating technology in near future. Chitosan is one of the most common biopolymer for the production of edible antioxidant film. In some recent studies, antioxidant chitosan based films were produced by incorporating green tea extract, vinyl alcohol, protocatechuic acid and essential oils [12-15]. All these studies proved that antioxidant films area promising material for food coating technology.

β-carotene is one of the natural carotenoids found in certain vegetables and fruits [16]. It is one of the precursors of vitamin A and is considered as an essential nutrient in human diet [17]. It also exhibits antioxidant [18] and anticancer activities [19]. To enhance its intake and stability in human diet many encapsulation methods have been developed [16]. Recent studies demonstrated that chitosan enhances the stability of β-carotene [20, 21].

This current study aimed (i) to produce chitosan from corpses of tree pest *H. abietis* for the first time, (ii) to prepare chitosan films with and without β-carotene, and (iii) to reveal antioxidant and antimicrobial properties of fully characterized films to see whether these edible and biodegradable films are applicable as food coating material.

2 Experimental

2.1 Materials

H. abietis samples were collected from the conifer woods on the campus of Aleksandras Stulginskis University, Kaunas, Lithuania (20.07.2016). Some *H. abietis* samples were kept in the Institute of Biology and Plant Biotechnology of Agronomy Faculty of Aleksandras Stulginskis University. *H. abietis* samples, that were used in the chitin extraction procedure, were washed with distilled water and dried at 50°C for a couple of days. DPPH$^{\bullet}$ (2,2-diphenyl-2-picrylhydrazyl hydrate, Sigma-Aldrich) was used in the antioxidant activity assay. Images of *H. abietis* and its damage on the coniferous plant are shown in Fig. 1.

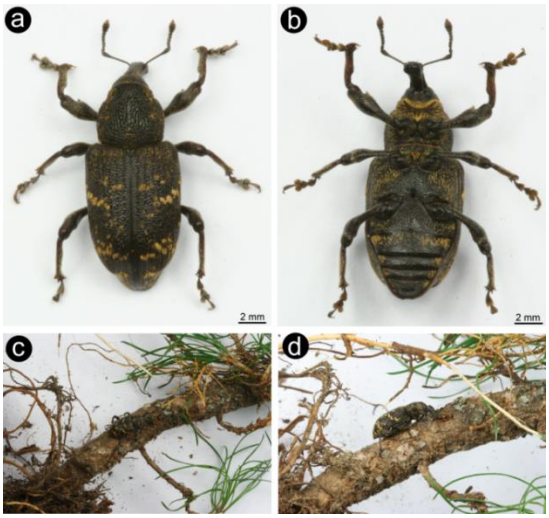

Figure 1: Habitus of *Hylobius abietis.* (a) dorsal, (b) ventral and (c, d) its damage on coniferous plant

2.2 Chitosan Production from H. abietis

H. abietis samples (100 g) were dried in the oven at 80°C for a week and ground to powder in a mortar. To get homogeneous size distribution, the samples were sieved with a 50 µm sieve. Chitosan was obtained after four steps; i.e., bleaching, demineralization, deproteinization, and deacetylation. The samples were treated in 0.5% NaOCl solution at room temperature for 1 h and recovered with a filter paper after extensive washing. Bleached samples were then transferred into acid solution (2 M HCl) and refluxed at room temperature to remove the minerals. Following the rinsing with water and filtration, the samples were subjected to alkaline solution (2 M NaOH) for 2 h to remove the proteins and then rinsed with water until neutrality. In the last step, the samples were deacetylated in hot alkaline solution of NaOH (60% w:w, at 100°C). After refluxing the sample for 4 h, the chitosan sample was rinsed with distilled water until neutrality. Finally, chitosan samples were separated by filtration and oven-dried at 50°C for a week. Chitin content of the insect and chitosan conversion yield were calculated on basis of the initial amount of the dried insect sample.

2.3 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectra of chitosan from *H. abietis* (10 mg) and commercial chitosan from Sigma-Aldrich (medium molecular weight, CAS Number 9012-76-4) were recorded on a Perkin-Elmer spectrophotometer in the wavenumber range of 4000-625 cm^{-1}. Deacetylation degree of chitosan from *H. abietis* was determined by using FT-IR data by the following Eq. [22];

$$DA = (A_{1655}/A_{3450}) \times (100/1.33) \quad (1)$$

2.4 Determination of the Molecular Weight

Molecular weight of chitosan, obtained from *H. abietis*, was measured by the Ubbelohde Dilution Viscometer. In the measurements, chitosan solutions with 5 different concentrations were prepared in the solvent systems of 0.1 M acetic acid + 0.2 M NaCl (1:1, v/v) at 25°C. Mark-Houwink equation was followed for molecular weight determination of the chitosan [23].

$$[\eta] = k\,Mv^{\alpha} \quad (2)$$

[η]: the intrinsic viscosity,

Mv: the viscosity-average molecular weight of the chitosan

k and α: Mark-Houwink-Sakurada constants ($k = 1.81 \times 10^{-3}$ and $\alpha = 0.93$).

2.5 Film Preparation

Two different films were prepared. For the first one, 10 mg chitosan and 50 µl glycerol were mixed in 1% acetic acid solution (10 ml) at room temperature for 2 days. For the second one, 10 mg chitosan, 50 µl glycerol and 0.1 mg β-carotene were mixed in 1% acetic acid solution (10 ml) at room temperature for 2 days. Then both gel materials were poured into Petri dishes (diameter: 5 cm) and incubated in an oven at 35°C for 3 days.

2.6 Surface Morphology of Chitosan, Chitosan Film, and Chitosan-β-Carotene Film

Surface morphology of chitosan, produced from *H. abietis*, was examined by SEM (FEG Quanta 250). Before getting the images, 15 mg of the chitosan was transferred onto a stub and coated with gold by a Gatan Precision Etching System (PECS). Surface characteristics of chitosan film and chitosan-β-carotene film were determined by AFM (BRUKER NanoScope V) under ambient conditions (24°C) using silicon nitride cantilevers. For both samples, 10 μm^2 areas were scanned. Also the root mean square roughness of the films was determined.

2.7 Film Thickness

A digital micrometer (Mitutoyo, China) was used to measure the thickness of the films. The measurements were carried out from 8 different parts of the films. For each film, the average values were calculated.

2.8 Transparency

A Shimadzu UV-3600 UV-VIS-NIR spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used to measure chitosan (from *H. abietis*) and chitosan-β-carotene composite films. The measurements were conducted at room temperature in the range of 400-700 nm and in the steps of 1 nm.

2.9 Mechanical Properties

Mechanical properties of the chitosan film and chitosan-β-carotene film were studied using a Material Testing Systems (MTS Insight 10) device (with a load cell of 250 N and deformation rate of 5 mm/min). MTS Test Works 4 software was used to estimate Elongation at break, Young modulus and tensile strength of the films. Samples measuring 5 mm in width and 40 mm in length were used in the measurements.

Incorporation of β-carotene into the chitosan film matrix increased the flexibility and decreased the light transmission. As it was reported before [24], flexible films can be used in the packaging technology. Additionally, lower transparency comparing to the control film will keep the antioxidant properties of the coated food by keeping the light transmittance and oxidation.

2.10 Water Solubility of the Films

The films were cut into pieces in sizes of 2 × 3 cm^2 to investigate water solubility of the films. The films were dried to constant weight at 60°C in the oven. Then the films were rested in 20 mL of water for 2 days. During the incubation the mixtures were stirred at different time intervals. Films were dried again to constant weight. The test was repeated three times and the % weight loss was determined by the following formula,

$$\%WL = \frac{(Wi - Wf)}{Wi} \times 100 \quad (3)$$

where *WL* stands for % weight loss, *Wi*, initial weight and *Wf* final weight of chitosan film.

2.11 Soil Solubility

Prior to soil solubility tests, chitosan films were cut into pieces (2 × 3 cm^2) and oven-dried at 60°C until constant weight. Dried films samples were placed into the soil in a depth of 15 cm. To maintain constant moisture, 10 mL of water was poured into the vessels each day during the test (15 days). Then, chitosan samples were recollected, cleaned and dried to constant weight. % weight loss in chitosan film samples was calculated using the formula (3), given in the previous section.

2.12 Antimicrobial Activity

Antimicrobial activity of chitosan obtained from *H. abietis* was evaluated by using disc diffusion method, and 28 bacteria strains were used in the experiments. Chitosan solutions (20 µL, 0.1 % w:w in 0.5% acetic acid solution) were pipetted onto sterile discs (diameter: 6 mm). After adjusting to 0.5 (10^6 cfu/mL) McFarland turbidity, fresh bacterial cultures (100 µL) were inoculated with sterile swab onto Mueller-Hinton Agar. Petri dishes were allowed to rest for 15 min at room temperature and then the discs with chitosan were placed onto the culture. Then, the cultures were kept in incubation for 24 h or 72 h at temperatures 30°C and 37°C. Inhibition zones appeared on the surface were measured in mm. Acetic acid solution (0.5% by volume) was used as negative control and kanamycin reference antibiotic disc was used as positive control under the same experimental conditions. Antimicrobial tests were performed in triplicate [25].

2.13 Antioxidant Activity Assay

Antioxidant activity assay of chitosan in gel and film forms and chitosan-β-carotene films was performed using DPPH radical scavenging method. In the assay a modified version of the method reported elsewhere [13] was followed and antioxidant activity experiments were done in triplicate. Briefly, chitosan gel (about 10 mg), pieces of chitosan or chitosan-β-carotene films weighing 10 mg were placed in tubes. Subsequently, following the addition of DPPH solution (6×10^{-5} M), the tubes were rested in darkness for 3 h at room temperature. Radical scavenging activity of the samples was determined spectrophotometrically by monitoring changes in colour at 517 nm on a UV-vis spectrophotometer (DU 730, Beckman Coulter).

Inhibition rate of each sample (%) was calculated using absorbance of the control and the sample according to the formula given below,

$$Inhibition\ (I\ \%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100 \quad (4)$$

36 mg of ABTS was dissolved in distilled water. In another beaker, 10 mL of potassium persulphate solution was prepared by dissolving 6.62 mg of potassium persulphate in distilled water. To activate the radicals, the two solutions were mixed together and incubated at room temperature for 12-16 h. The activated radical solution was diluted until giving 1.5 absorbance at 734 nm on UV-vis spectrophotometry (DU 730, Beckman Coulter). This absorbance measurement was also recorded as the absorbance reading for the control. Chitosan samples weighing 10 mg were cut out from chitosan films. Then, the chitosan samples were kept in 1 mL of radical solution for 30 minutes. Finally, absorbance readings were done at 734 nm. The ABST radical scavenging activity of the samples was calculated using the following equation:

$$\text{Inhibition (I\%)} = ((A_{control} - A_{sample}) / A_{control}) \times 100 \quad (5)$$

The same procedure was followed for trolox with varying concentration i.e., 25-100 µg/mL to draw a concentration-absorbance plot. Then, by using the calibration curve, trolox concentrations were obtained.

3 Results and Discussion

3.1 ATR-FTIR Spectrum Analysis of H. abietis Chitosan

Chemical composition of the chitosan from the exoskeleton of *H. abietis* and the commercial chitosan was examined by FTIR spectrum analysis (Fig. 2). Other than the characteristics absorbance peaks, two sharp peaks were observed at 1647 cm^{-1} and 1579 cm^{-1} in the spectrum of *H. abietis* chitosan and at 1651 cm^{-1} and 1565 cm^{-1} for the commercial chitosan. Earlier studies on FTIR spectrum analysis of chitosan have demonstrated that band stretching of C = O at 1650 cm^{-1} (Amide I band) and stretching of NH_2 at 1590 cm^{-1} (Amide II band) are considered as an indication of chitosan [26]. As mentioned above, presence of those sharp peaks demonstrates that *H. abietis* chitin was converted into chitosan. Also, intensity of the peaks indicates deacetylation degree of chitosan. The deacetylation degree of *H. abietis* chitosan was calculated as 81.3% based on FTIR spectrum. This high conversion rate indicates that the method used in chitosan production was effective. Overall FTIR spectrum of *H. abietis* chitosan bears a high similarity to that of the commercial chitosan and other chitosan samples obtained from other organisms [27,28]. Molecular weight of the chitosan was calculated as 7.3 kDa, which is characterized as low molecular weight chitosan [29]. Chitin content of *H. abietis* was found as 27.9% chitin, and 86.2% of chitin was converted into chitosan.

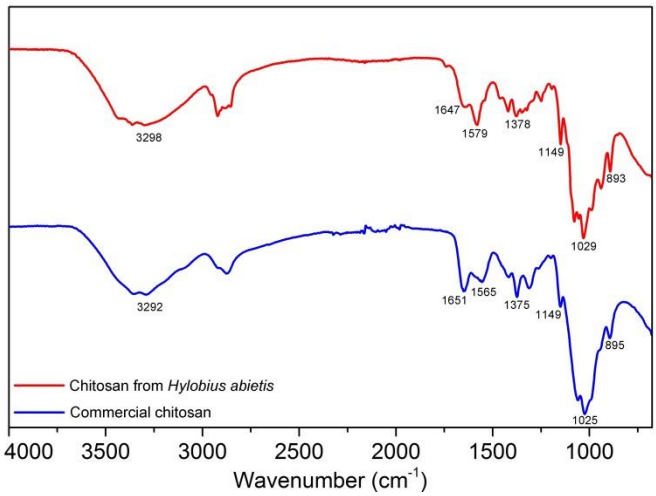

Figure 2: FT-IR spectra of the chitosan obtained from *Hylobius abietis* and the commercial chitosan from the shrimp

3.2 Surface Characteristics

SEM analysis showed that surface of *H. abietis* chitosan was smooth at the magnifications 1.000X and 5.000X (Figs. 3(a), 3(b)). However, at higher magnifications (10.000X and 20.000X), the nanofibers on the chitosan surface were clearly visible (Figs. 3(c), 3(d)). Also, the pores were present in some parts (Fig. 3(d)). Rough surface morphologies were observed on both chitosan film and chitosan-β-carotene film (Figs. 3(e), 3(f)). AFM analysis revealed that surface morphologies of chitosan film and chitosan-β-carotene film were almost the same with slightly rough structure (Fig. S1). The root mean square roughness values were recorded as 86.5 nm for chitosan film and 78.4 nm for chitosan-β-carotene film.

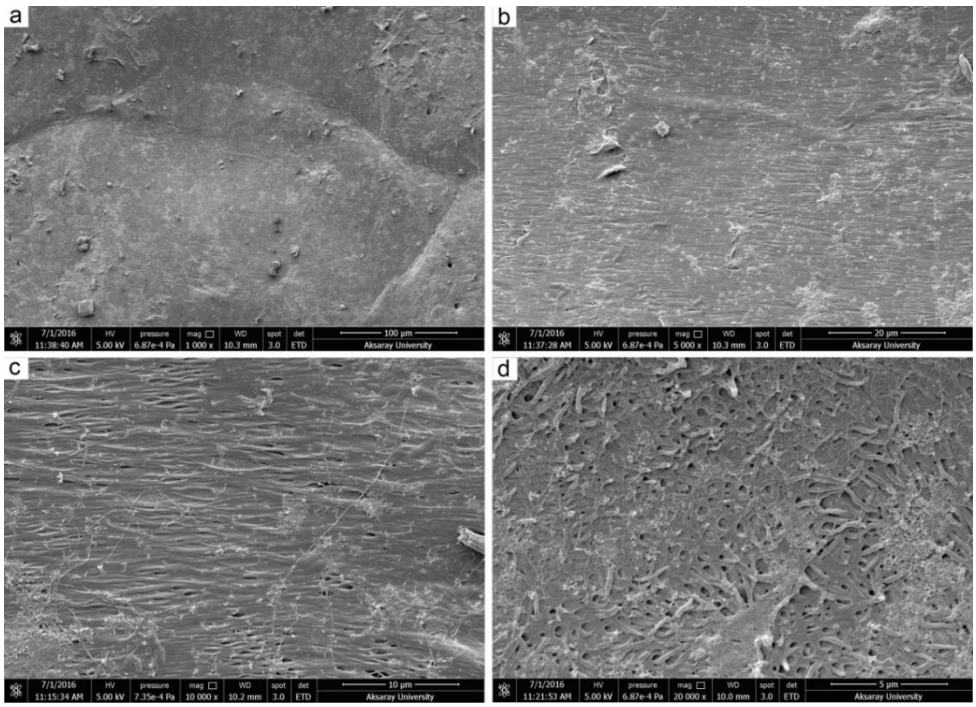

Figure 3: Scanning electron microscopy (SEM) images of (a,b,c,d) chitosan from *Hylobius abietis*, (e) chitosan film and (f) chitosan-β-carotene film

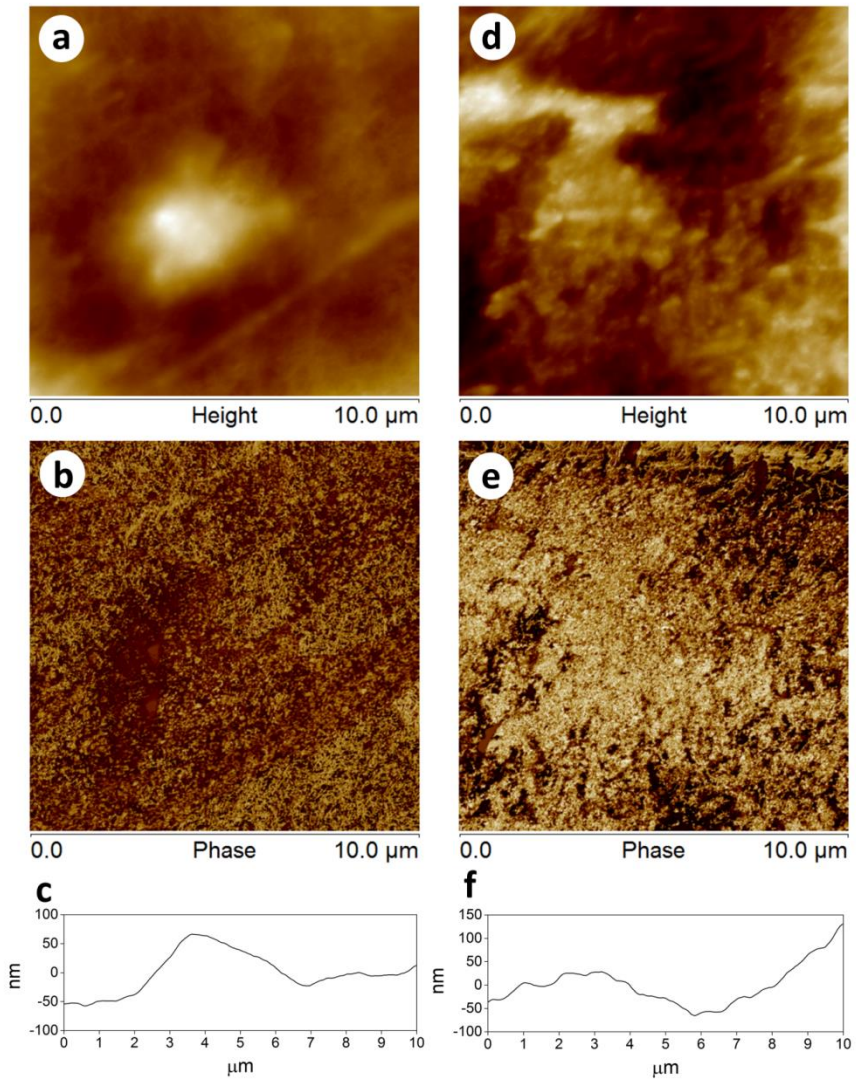

Figure S1: Atomic-force microscopy (AFM) images and the mean squared roughness of (a,b,c) chitosan films and (d,e,f) chitosan-β-carotene films

3.3 Film Thickness

The average thickness of chitosan and chitosan-β-carotene films was recorded as 0.044 ± 0.001 mm and 0.045 ± 0.002 mm, respectively. As it is seen from the average values, there was not a big difference in thickness of the films.

3.4 Transparency

Transparency is a very important characteristic of the films for determining the application area of material. The light transmittance results of the produced films are shown in Figs. 4(a) and 4(b). The transparency of the insect chitosan film was determined as 74.4 at 700 nm, 70.1 at 600 nm, 63.1 at 500 nm and 50.0 at 400 nm, while the transparency of β-carotene included insect chitosan film was recorded as 52.4 at 700 nm, 48.3 at 600 nm, 42.6 at 500 nm and 32.9 at 400 nm. As expected, addition of β-carotene into the chitosan film reduced the transparency, because the colour of the film was changed slightly from white to orange and this little change in the colour decreased the light transmission. Visual transparency of the films is shown in Figs. 4(c), 4(d). As depicted in Figs. 4(c) and 4(d), the colour of the

chitosan films was turned into slightly reddish following the β-carotene incorporation, but they were still transparent.

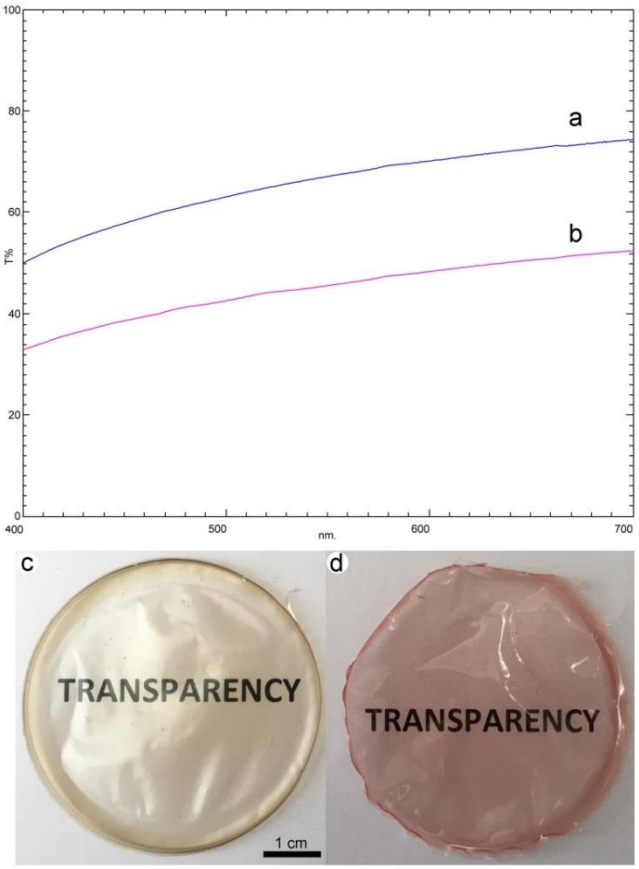

Figure 4: Optical transmittance: (a) chitosan film, obtained from *Hylobius abietis* and (b) chitosan-β-carotene film. Visual transparency: (c) chitosan film, obtained from *Hylobius abietis* and (d) chitosan-β-carotene film

Light transmission of low molecular weight chitosan films (400-600nm) were recorded between 50 and 90%, depending on the concentration of the plasticizer that was used [30]. In some previous studies, light transmission values of some edible films were determined and the results are; between 40% and 85% for chitosan-nanofibrillated cellulose film [31], more than 80% for fish gelatin-chitosan blend film [32], and around 60% for chitosan-poly (vinyl alcohol) films [13]. Considering the obtained results in these earlier studies, light transmission values of the chitosan and chitosan-β-carotene films in the present study were found almost similar with them.

3.5 Mechanical Properties

Tensile strength of the chitosan film and chitosan-β-carotene film was measured as 9.27 ± 0.87 MPa and 6.82 ± 0.61 MPa respectively. Elongation at break was recorded as 53.84 ± 5.47% for chitosan film and 62.92 ± 5.44% for chitosan-β-carotene film. Young Modulus of the chitosan film and chitosan-β-carotene film were determined as 96.20 ± 9.22 MPa and 70.41 ± 2.57 MPa, respectively (Fig. 5). Incorporation of β-carotene into chitosan film decreased Young Modulus and tensile strength and increased elongation at break. In an earlier study, chitosan films with a mixture of soy protein and whey protein with β-carotene were produced. Young Modulus of soy protein β-carotene and whey protein β-carotene were observed as 109 MPa and 50.3 MPa respectively. In the same regard, elongation at break of soy protein β-carotene and whey protein β-carotene was recorded as 76% and 6% respectively [33].

Considering these results, Young Modulus and elongation at break values of the chitosan-β-carotene film were found higher than whey protein β-carotene film and closer to soy protein β-carotene film.

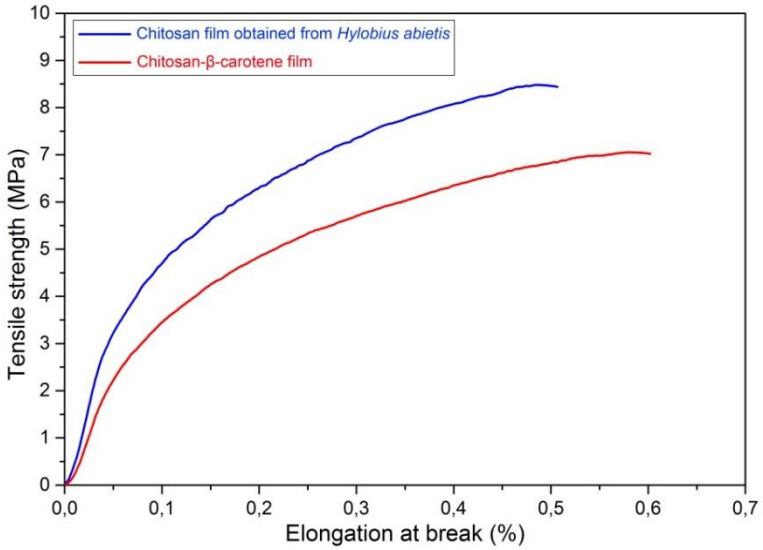

Figure 5: Mechanical properties of the chitosan film from *Hylobius abietis* and chitosan-β-carotene film

3.6 Water and Soil Solubility of the Films

It was observed that the control and chitosan-β-carotene films retained their integrity at the end of two-day incubation in water. The results showed that water solubility was reduced when beta carotene was added into the chitosan film. Therefore, the films resistance was increased against water. The water solubility values for chitosan control and chitosan-β-carotene films were recorded as 28.4 ± 1.21% and 31.1 ± 1.34%, respectively. For soil solubility, weight loss values of chitosan control and chitosan-β-carotene films were observed as 30.4% and 23.5%, respectively.

3.7 Antioxidant Activity

DPPH absorption inhibition (%) of the chitosan gel, chitosan film and chitosan-β-carotene film were recorded as 8.99%, 72.87% and 81.57%, respectively. As expected, the chitosan gel exhibited comparatively low antioxidant activity. This low activity can be ascribed to the high water content of the chitosan gel and therefore lower concentration. Incorporation of the β-carotene into chitosan almost completely inhibited DPPH absorption by exceeding that of chitosan about 9%. As reported previously, β-carotene has high antioxidant activity and scavenges free radicals [34].

In a previous study, DPPH radical scavenging activity of protocatechuic acid (a natural phenolic substance) incorporated in chitosan films was recorded in the range of 14-45% [14]. In another study, pure chitosan film showed inhibition value as 43.07%, while antioxidant activity of films revealed a gradual decrease (34-24.82%) with the addition of poly vinyl alcohol [13]. As can be seen, the inhibition values of the chitosan film and chitosan-β-carotene film, obtained from *H. abietis*, were significantly higher than the previous studies. For this reason, the chitosan-β-carotene film produced in the present study may be called as an antioxidative film.

The ABST radical scavenging activity of the chitosan and chitosan-beta carotene films was recorded as 74.38% and 76.55%. Similarly, TE activity of the chitosan film was found as 176.40 µg/mg and chitosan-beta carotene films had TE activity of 181. 63 µg/mg. This shows that the inclusion of the beta-carotene enhanced the antioxidant activity of the chitosan.

3.8 Antimicrobial Activity of H. abietis Chitosan

The antimicrobial activity of the chitosan produced from *H. abietis* was studied by following the disc diffusion method and 28 bacterial strains were used in the experiments. Acetic acid solution of the chitosan (1 g of chitosan was dissolved in 1% acetic acid solution) was used in the antimicrobial activity experiments. The antibiotic kanamycin was used as a control and the data obtained is presented in Fig. 6. Chitosan exhibited antibacterial activity on 18 strains out of 28. Highest activity was recorded against *V. parahaemolyticus* and followed by *A. baumannii* and *Streptococcus pneumonia*. Chitosan showed a comparatively lower activity on *Klebsiella pneumoniae subsp. pneumoniae ATCC 700603* and *S. sonnei*. Kanamycin, on the other hand, exhibited a much higher antibacterial activity on all the strains (2-5 times of that of the chitosan); and this can be attributed to the toxicity of the antibiotic kanamycin. Yet, considering its nontoxic nature, antibacterial studies with the chitosan can be fruitful.

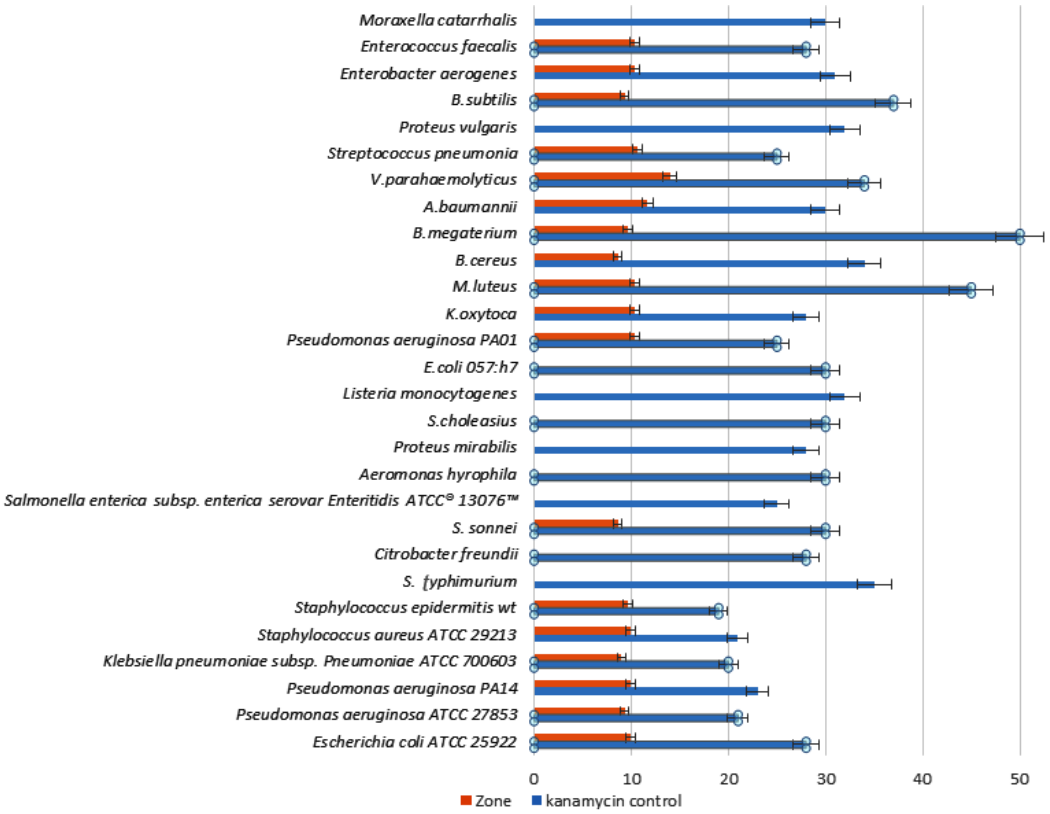

Figure 6: Antimicrobial activity of the chitosan gel obtained from *Hylobius abietis*

4 Conclusions

Here, a common plant parasitic insect (with its huge amount of waste biomass) was converted into versatile biopolymer (chitosan) successfully. With high chitin contents and chitosan yield, *H. abietis* could be used as a source for chitin and chitosan production. Also the obtained chitosan was used for production of the chitosan based film, exhibiting high antioxidative and antimicrobial activities. These chitosan and chitosan-β-carotene films, obtained from *H. abietis*, can find applications in food industry as a coating material thanks to their biodegradable, antioxidant and antimicrobial properties.

References

1. Örlander, G., Nordlander, G. (2003). Effects of field vegetation control on pine weevil (*Hylobius abietis*) damage to newly planted Norway spruce seedlings. *Annals of Forest Science*, *60*, 667-671.

2. Wainhouse, D., Staley, J., Johnston, J., Boswell, R. (2005). The effect of environmentally induced changes in the bark of young conifers on feeding behaviour and reproductive development of adult *Hylobius abietis* (Coleoptera: Curculionidae). *Bulletin of Entomological Research, 95*, 151-159.
3. Dillon, A. B., Ward, D., Downes, M. J., Griffin, C. T. (2006). Suppression of the large pine weevil *Hylobius abietis* (L.)(Coleoptera: Curculionidae) in pine stumps by entomopathogenic nematodes with different foraging strategies. *Biological Control, 38,* 217-226.
4. Day, K., Nordlander, G., Kenis, M., Halldorson, G. (2007). General biology and life cycles of bark weevils. *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis*. Springer, Netherland, 331-349.
5. Williams, C. D., Dillon, A. B., Harvey, C. D., Hennessy, R., Mc Namara, L. et al. (2013). Control of a major pest of forestry, Hylobius abietis, with entomopathogenic nematodes and fungi using eradicant and prophylactic strategies. *Forest Ecology and Management, 305,* 212-222.
6. Ziogas, A., Valenta, V., Paskevicius, H., Posiunas, R. (1976). Chemical control of the large pine weevil in the Lithuanian SSR. *Acta Enterologica Latinoamericana, 3,* 101-111.
7. Voolma, K., Sibul, I. (2006). The effect of collecting fluid on the catch results of *Hylobius abietis* in pitfall traps. *The Effect of Collecting Fluid on the Catch Results of Hylobius Abietis in Pitfall Traps, 4,* 457-460.
8. Anitha, A., Sowmya, S., Kumar, P. S., Deepthi, S., Chennazhi, K. et al. (2014). Chitin and chitosan in selected biomedical applications. *Progress in Polymer Science, 39,* 1644-1667.
9. Suh, J. K. F., Matthew, H. W. (2000). Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. *Biomaterials, 21,* 2589-2598.
10. Dash, M., Chiellini, F., Ottenbrite, R., Chiellini, E. (2011). Chitosan-A versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science, 36,* 981-1014.
11. Jadhav, S., Nimbalkar, S., Kulkarni, A., Madhavi, D. (1995). Lipid oxidation in biological and food systems. *Food Science and Technology.* Marcel Dekker. 5-64.
12. Ballester-Costa, C., Sendra, E., Fernández-López, J., Viuda-Martos, M. (2016). Evaluation of the antibacterial and antioxidant activities of chitosan edible films incorporated with organic essential oils obtained from four *Thymus* species. *Journal of Food Science and Technology, 53,* 3374-3379.
13. Hajji, S., Chaker, A., Jridi, M., Maalej, H., Jellouli, K. et al. (2016). Structural analysis, and antioxidant and antibacterial properties of chitosan-poly (vinyl alcohol) biodegradable films. *Environmental Science and Pollution Research, 23,* 15310-15320.
14. Liu, J., Meng, C. G., Liu, S., Kan, J., Jin, C. H. (2017). Preparation and characterization of protocatechuic acid grafted chitosan films with antioxidant activity. *Food Hydrocolloid, 63,* 457-466.
15. Siripatrawan, U., Noipha, S. (2012). Active film from chitosan incorporating green tea extract for shelf life extension of pork sausages. *Food Hydrocolloid, 27,* 102-108.
16. Donhowe, E. G., Kong, F. (2014). Beta-carotene: digestion, microencapsulation, and in vitro bioavailability. *Food Bioprocess Technology, 7,* 338-354.
17. Yi, J., Li, Y., Zhong, F., Yokoyama, W. (2014). The physicochemical stability and in vitro bioaccessibility of beta-carotene in oil-in-water sodium caseinate emulsions. *Food Hydrocolloid, 35,* 19-27.
18. Yi, J., Lam, T. I., Yokoyama, W., Cheng, L. W., Zhong F. Beta-carotene encapsulated in food protein nanoparticles reduces peroxyl radical oxidation in Caco-2 cells. *Food Hydrocolloid, 43,* 31-40.
19. Gloria, N. F., Soares, N., Brand, C., Oliveira, F. L., Borojevic, R. et al. (2014). Lycopene and beta-carotene induce cell-cycle arrest and apoptosis in human breast cancer cell lines. *Anticancer Research, 34,* 1377-1386.
20. Hou, Z., Gao, Y., Yuan, F., Liu, Y., Li, C. et al. (2010). Investigation into the physicochemical stability and rheological properties of β-carotene emulsion stabilized by soybean soluble polysaccharides and chitosan. *Journal of Agricultural and Food Chemistry, 58,* 8604-8611.
21. Hou, Z., Zhang, M., Liu, B., Yan, Q., Yuan, F. et al. (2012). Effect of chitosan molecular weight on the stability and rheological properties of β-carotene emulsions stabilized by soybean soluble polysaccharides. *Food Hydrocolloid, 26,* 205-211.
22. Khan, T. A., Peh, K. K., Ching, H. S. (2002). Reporting degree of deacetylation values of chitosan: the influence of analytical methods. *Journal of Pharmacy and Pharmaceutical Sciences, 5,* 205-212.

23. Roberts, G. A., Domszy, J. G. (1982). Determination of the viscometric constants for chitosan. *International Journal of Biological Macromolecules, 4,* 374-377.
24. Arrieta, M. P., López, J., Hernández, A., Rayón, E. (2014). Ternary PLA-PHB-Limonene blends intended for biodegradable food packaging applications. *European Polymer Journal, 50,* 255-270.
25. Gupta, D., Dubey, J., Kumar, M. (2016). Phytochemical analysis and antimicrobial activity of some medicinal plants against selected common human pathogenic microorganisms. *Asian Pacific Journal of Tropical Disease, 6,* 15-20.
26. Moreno-Vásquez, M. J., Valenzuela-Buitimea, E. L., Plascencia-Jatomea, M., Encinas-Encinas, J. C., Rodríguez-Félix, F. et al. (2017). Functionalization of chitosan by a free radical reaction: Characterization, antioxidant and antibacterial potential. *Carbohydrate Polymers, 155,* 117-127.
27. Hajji, S., Younes, I., Ghorbel-Bellaaj, O., Hajji, R., Rinaudo, M. et al. (2014). Structural differences between chitin and chitosan extracted from three different marine sources. *International Journal of Biological Macromolecules, 65,* 298-306.
28. Paulino, A. T., Simionato, J. I., Garcia, J. C., Nozaki, J. (2006). Characterization of chitosan and chitin produced from silkworm crysalides. *Carbohydrate Polymers, 64,* 98-103.
29. Goy, R. C., Britto, D. D., Assis, O. B. (2009). A review of the antimicrobial activity of chitosan. *Polimeros, 19,* 241-247.
30. Leceta, I., Guerrero, P., De la Caba, K. (2013). Functional properties of chitosan-based films. *Carbohydrate Polymers, 93,* 339-346.
31. Fernandes, S. C., Freire, C. S., Silvestre, A. J., Neto, C. P., Gandini, A. et al. (2010). Transparent chitosan films reinforced with a high content of nanofibrillated cellulose. *Carbohydrate Polymers, 81,* 394-401.
32. Hosseini, S. F., Rezaei, M., Zandi, M., Ghavi, F. F. (2013). Preparation and functional properties of fish gelatin-chitosan blend edible films. *Food Chemistry, 136,* 1490-1495.
33. López-Rubio, A., Lagaron, J. M. (2011). Improved incorporation and stabilisation of β-carotene in hydrocolloids using glycerol. *Food Chemistry, 125,* 997-1004.
34. Mueller, L., Boehm, V. (2011). Antioxidant activity of β-carotene compounds in different *in vitro* assays. *Molecules, 16,* 1055-1069.