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Cent. Eur. J. Chem. • 11(7) • 2013 • 1066-1078
DOI: 10.2478/s11532-013-0241-4

Central European Journal of **Chemistry**

Development of antimicrobial packaging materials with immobilized glucose oxidase and lysozyme

Research Article

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Received 2 December 2012; Accepted 18 February 2013

Abstract: Packaging based on immobilization of antimicrobial enzymes provides a promising form of active packaging systems applicable in food processing. Glucose oxidase and lysozyme were immobilized by the Ugi reaction with cyclohexyl isocyanide and glutaraldehyde on polyamide and ionomer films partially hydrolysed by hydrochloric acid. The immobilization of the enzymes on the surface of films was confirmed by FT-IR spectroscopy and the films were characterized by the specific activity of the immobilized enzymes. The enzyme migration into model solutions and the effect of pH, temperature and storage time on the activity of immobilized enzyme were also evaluated. Immobilization of lysozyme onto polyamide and ionomer films resulted in the loss of enzyme activity. The polyamide and ionomer films with immobilized glucose oxidase inhibited the growth of bacteria *Escherichia coli* CNCTC 6859, *Pseudomonas fluorescens* CNCTC 5793, *Lactobacillus helveticus* CH-1, *Listeria ivanovii* CCM 5884 and *Listeria innocua* CCM 4030 on agar media.

Keywords: *Antimicrobial packaging • Immobilization • Glucose oxidase • Lysozyme*

1. Introduction

The utilization of immobilization of bioactive compounds onto polymer surfaces has grown rapidly in the past decade in various industries such as biomedical, textile, microelectronic, bioprocessing and food packaging [1,2]. Packaging based on immobilization of antimicrobial enzymes provides a promising form of active packaging systems applicable in food processing for prolongation of shelf-life of non-sterile, chilled or minimally processed foods. Many concepts concerning not only immobilization of antimicrobial enzymes (*e.g.* glucose oxidase, lysozyme) but also enzymes suitable for „in-package" processing and production of functional foods (*e.g.* naringinase, invertase, lactase, cholesterol reductase) have recently been described [3-17].

Many methods such as adsorption, ionic binding, covalent binding by chemical coupling, crosslinking, entrapment, encapsulation or graft co-polymerization have been developed for immobilization of enzymes onto a wide variety of solid supports [9,18-20]. The covalent binding of biocatalysts on a solid support is one of the most extensively used methods of enzyme immobilization [7]. However, the structure of traditional polymers used in food packaging is commonly inert and their surface must be activated via suitable chemical reagents or functionalized before immobilization [1,2,9,18,19].

Covalent immobilization offers several advantages, especially in preservation of enzyme activity. Enzymes can be sensitive to processing conditions due to the dependence of their activity on pH, temperature and presence of salts, *etc.* [14]. Covalent binding provides

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higher enzyme stability over a wider temperature and pH range and higher storage stability or enables repeated use of immobilized enzymes. Moreover in the case of active food packaging applications, a covalent linkage ensures that the bioactive compound should not migrate to the food and thus may offer the regulatory advantage of not being considered as a food additive [1,3,7]. Conversely, the total activity of covalently immobilized enzyme is lower than the activity of free enzyme [1,9,18,21].

Antimicrobial enzymes are ubiquitous in nature, playing a significant role in the defense mechanisms of living organisms against infection caused by bacteria and fungi. Two classes of enzymes are relevant, namely the hydrolases capable of degrading the key structural components of the cell walls of bacteria and/or fungi, and the oxidoreductases generating *in situ* reactive molecules with antimicrobial activity [21,22]. However, the application of these enzymes in food preservation, namely packaging, still remains more theoretical rather than practical.

Lysozyme (LYS; N-acetylhexosaiminidase) from hen egg white is hydrolase consisting of 129 amino acids and four disulfide bonds. It lyses the cell wall of certain species of bacteria by hydrolyzing the β(1→4)-glucosidic linkages of the peptidoglycan. LYS is active mainly on Gram-positive bacteria because they lack an outer membrane. Gram-negative bacteria are more sensitive to LYS in combination with certain physical treatments or chemicals (*e.g.* EDTA). Hen egg white lysozyme is utilized as a food preservative (E 1105) with antimicrobial effects preventing "gas blowing" from growth of *Clostridium tyrobutyricum* in ripened cheeses and inhibiting lactic acid bacteria in wines. It can be used to preserve seafoods, vegetables, pasta and salads [7,21-24].

The glucose oxidase (GOX) is oxidoreductase produced by molds such as *Aspergillus niger* and *Penicillium* spp. It is a dimeric protein with a molecular weight of 140-160 kDa containing non-covalently bound flavin adenine dinucleotide (FAD) per monomer as a cofactor. GOX catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone and H_2O_2. The antimicrobial activity of GOX is attributed to the toxicity of formed H_2O_2, although the lowering of pH by the production of D-gluconic acid from D-glucono-δ-lactone and H_2O may also influence the growth of microorganisms. Thus, the GOX system shows antibacterial activity towards both Gram-negative and Gram-positive bacteria. But the susceptibility of various microorganisms towards the GOX system is also dependent on their ability to produce H_2O_2 scavengers such as catalase, glutathione or ascorbic acid. GOX in the food industry can be used for the prevention of non-enzymatic browning in egg white powder or potato products (chips); removal of oxygen preventing rancidity in oils or inhibiting growth of aerobic microorganisms in packages (oxygen scavenger); prevention of off-flavours and colour changes in fruit juices, wine and beer etc. or improvement of the quality and the baking properties of dough [21,22,25,26]. Glucose oxidase is not listed as a food additive in the European Union (EU) food legislation, but it can be used as a processing aid under the existing national legislation of the Member States, until the EU list of approved food enzymes has been drawn up (Regulation (EC) No 1332/2008).

The aim of this study was to (i) prepare polymer films with immobilized glucose oxidase and lysozyme and evaluate their enzyme activity under various conditions (pH, temperature), (ii) assess the stability of the enzyme bonding to the polymers and (iii) test antimicrobial efficiency of prepared films against selected indicator microorganisms .

2. Experimental procedure

2.1. Packaging materials

Polyamide film CPA 25 (PA 6, thickness 25 μm, MF FOLIEN, Germany); ionomer film DuPont™ Surlyn® 8140 (ethylene/methacrylic acid copolymer containing 19% of methacrylic acid, thickness 80 μm, DuPont de Nemours International S.A., Switzerland) and regenerated (uncoated) cellulose film NatureFlex™ (thickness 25 μm, Innovia Films, United Kingdom) were kindly donated by the industrial partners.

2.2. Chemicals

Glucose Oxidase (GOX; β-D-glucose:oxygen l-oxidoreductase, E.C. 1.1.3.4, from *Aspergillus niger* - Type II, ≥15,000 units (U) g^{-1} solid, without added oxygen); lysozyme (LYS; Mucopeptide *N*-acetylmuramoylhydrolase, E.C.3.2.1.17, from chicken egg white, dialyzed, lyophilized, powder, ~100000 units (U) mg^{-1}); *Micrococcus lysodeikticus* ATCC No. 4698 (suitable as substrate for the assay of lysozyme, lyophilized cells); glutaraldehyde solution (technical, 50% in H_2O (5.6 mol L^{-1})); cyclohexyl isocyanide (98%) and potassium phosphate monobasic (p.a., anhydrous, ≥99.0%) were purchased from Sigma Aldrich (Czech Republic). D-glucose (monohydrate, p.a.) was obtained from Lach-Ner s.r.o. (Czech Republic).

2.3. Immobilization of glucose oxidase and lysozyme onto polymer films

The circular samples of polyamide and ionomer films (area 16 cm^2) were partially hydrolysed with hydrochloric acid (3 mol L^{-1}) for 20 minutes, then thoroughly washed with distilled water and dried in air. A solution (160 µL) of glucose oxidase (concentration 24.8, 248 and 2480 U mL^{-1}) or lysozyme (concentration 40 000, 400 000, 4 000 000 U mL^{-1}) was applied onto a glass Petri dish, then an aqueous solution of glutaraldehyde (2.5%, 40 µL) and undiluted cyclohexyl isocyanide (16 µL) were added and mixed together. Hydrolysed films were placed onto the reaction mixture in order to create a continuous, uniform layer under the film. The samples were incubated at 4°C for 2-10 days. After incubation the films were thoroughly washed three times for 20 minutes in potassium phosphate buffer (0.1 mol L^{-1}, pH = 7). Slightly wet samples were stored at 4°C.

Cellulose film with absorbed enzyme was also prepared for the evaluation of efficiency of free enzyme on microorganisms inhibition. The circular samples of the film (area 16 cm^2) were immersed into a solution (20 mL) of glucose oxidase and lysozyme with concentrations mentioned above at 4°C for 1 hour. The samples were dried in the air until they were slightly wet and stored at 4°C.

2.4. Fourier transform infrared (FTIR) spectroscopy

Formation of covalent bonds between the enzymes and packaging materials was assessed by FTIR spectroscopy. The infrared spectra were scanned in spectral range 4000 – 400 cm^{-1} by FTIR spectrometer Nicolet 6700 (Thermo-Nicolet, USA) in reflectance mode with detector DTGS and Attenuated Total Reflectance (GladiATR) accessory with diamond interface. Parameters of measurement were: resolution 4 cm^{-1}; spectral acumulation 64; apodization Happ-Genzel. Spectra were processed by software Omnic 7.3 (Nicolet Instruments Co., USA).

2.5. Determination of glucose oxidase activity

The activity of glucose oxidase was determined by Megazyme Assay Kit Glucose Oxidase K-GLOX 01/05 (Megazyme International Ireland Ltd., Ireland) based on the catalysis of oxidation of β-D-glucose to D-glucono-δ-lactone with the concurrent release of hydrogen peroxide. The solution (2.0 mL) of potasium phosphate buffer (60 mmol L^{-1}, pH = 7) containing peroxidase (0.64 U mL^{-1}), p-hydroxybenzoic acid (12 mmol L^{-1}) and aminoantipyrine (0.4 mmol L^{-1}) was mixed with β-D-glucose (0.5 mol L^{-1}, 0.5 mL). The mixture in the cuvette was equilibrated to 25°C and the absorbance (A_1) at 510 nm was measured by Lambda 25 UV/VIS spectrophotometer (PerkinElmer, USA) after 5 minutes. Then the reaction was started by the addition of film (1 cm^2) with immobilized GOX or enzyme solution (0.5 mL). The samples were shaken (1 shake s^{-1}) in a water bath GFL 1083 (GFL, Germany) at 25°C during incubation time and the absorbance (A_2) of a formed red quinoneimine dye was read after exactly 20 minutes. Polymer that did not contain immobilized GOX served as control sample (blank).

Considering that the standard curve relating glucose oxidase activity (mU/assay) to absorbance increase in 20 minutes at 510 nm is not perfectly linear, the activity of immobilized GOX was calculated by the equation of the standard curve given in the kit instruction that was adapted for determination of activity of GOX immobilized on polymer films (Eq. 1):

$$Activity\ (mU/cm^2) = 15.4 \cdot (\Delta A_{SAMPLE,510nm} - \Delta A_{BLANK,510nm})^2 + 44.7 \cdot (\Delta A_{SAMPLE,510nm} - \Delta A_{BLANK,510nm}) + 0.03 \quad (1)$$

where ΔA_{510nm} is absorbance difference (A_2-A_1) for both blank and sample.

2.6. Determination of lysozyme activity

The activity of lysozyme was determined according to Sigma quality control test procedure based on the method of Shugar [27]. The suspension (2.5 mL) containing lyophilized *M. lysodeikticus* cells (0.015% w/v) in potassium phosphate buffer (66 mmol L^{-1}, pH = 6.24, initial absorbance of 0.6-0.7 at 450 nm) was equilibrated to 25°C. Polymer (1 cm^2) with immobilized LYS or enzyme solution (0.1 mL) was added into the cell suspension in a water bath. The decrease in absorbance of the suspension at 450 nm attributed to the lysis of *M. lysodeikticus* cells by LYS was monitored with UV/VIS spectrophotometer Spekol® 1300 (Analytik Jena AG, Germany) with cell holder thermostatted with thermostat GD 120 (Grant Instruments Ltd., UK) for 5 minutes. *M. lysodeikticus* incubated with polymer that did not contain immobilized LYS served as control sample (blank). The activity of immobilized LYS was calculated by Eq. 2:

$$Activity\ (units/cm^2) = \frac{(\Delta A_{SAMPLE,450nm}/\min - \Delta A_{BLANK,450nm}/\min) \cdot d}{(0.001) \cdot S} \quad (2)$$

where $\Delta A_{450nm}/min$ is maximum linear rate for both the sample and blank obtained from absorbance vs time curve; *d* is dilution factor; *S* (cm^2) is area of the polymer film; value *0.001* is change in absorbance at A_{450nm} as per the unit definition. One unit is defined as decrease

of absorbance at 450 nm by 0.001 per minute using a suspension of *M. lysodeikticus* (0.015%) at pH = 6.24 and 25°C.

2.7. Migration of immobilized enzymes

The migration of glucose oxidase and lysozyme from the films into model solutions was studied under the following conditions: the film (4 cm^2) was placed into vials with potassium phosphate buffer (0.1 mol L^{-1}, 4 mL, pH = 7.0) and aqueous solutions of potassium dihydrogen phosphate (0.1 mol L^{-1}, 4 mL, pH = 4.5) and acetic acid (3% w/v, 4 mL, pH = 2.5). The vials were shaken (1 shake s^{-1}) in a water bath at 23°C for 168 hours. Samples of solution with glucose oxidase (0.5 mL) and lysozyme (0.1 mL) were taken with a pipette after 48 and 168 hours for the determination of activity of migrating enzyme by the methods mentioned above.

2.8. Characterization of the prepared films

The effect of pH on the activity of immobilized enzyme was evaluated. After migration into model solutions with pH = 2.5, 4.5 and 7.0 for 168 hours enzyme activity of films was measured and compared with the activity of the films at the beginning of experiment. The retention of enzyme activity was also observed during the storage of prepared films at 4°C during 240 days.

The influence of temperature on the stability of immobilized enzymes was assessed. The films with immobilized enzyme (4 cm^2) were placed into vials with potassium phosphate buffer (0.1 mol L^{-1}, 6 mL, pH = 7) and heated in a water bath at 40, 50 and 60°C respectively. The activity of immobilized enzyme was determined after 1, 3 and 5 hours and compared with activity of free enzyme (100 mU mL^{-1}) in potassium phosphate buffer (0.1 mol L^{-1}) after heat treatment.

2.9. Efficiency of the films against microorganisms

The antimicrobial activity of films was tested against bacteria *Escherichia coli* CNCTC 6859, *Pseudomonas fluorescens* CNCTC 5793, *Lactobacillus helveticus* CH-1, *Bacillus subtilis* CNCTC 5615, *Listeria ivanovii* CCM 5884 and *Listeria innocua* CCM 4030.

The agar media (250 mL) were inoculated with suspension (1 mL) of indicator microorganisms containing 10^5–10^6 CFU mL^{-1}. Samples of the prepared films (4.0 cm^2) were placed with the active surface down on (i) PCA agar (Merck KGaA, Germany) inoculated with *Bacillus subtilis* or *Pseudomonas fluorescens*; (ii) VRBD agar (Merck KGaA, Germany) inoculated with *Escherichia coli*; (iii) MRS agar (Merck KGaA, Germany) with *Lactobacillus helveticus*; (iv) PALCAM agar (Merck KGaA, Germany) with *Listeria ivanovii* or *Listeria innocua*. The Petri dishes with tested films were incubated at 37°C for *E.coli, L. ivanovii* and *L. innocua,* at 30°C for *B. subtilis* and *P. fluorescens* and at 42°C for *L. helveticus* for 24-48 hours. Then the inhibitory zones formed round the film samples were evaluated.

The antimicrobial activity of the films was also tested against psychrophilic bacteria *L. ivanovii* and *L. innocua* during cold storage at 4°C. Retention of enzyme activity was observed by repeated use of the films during antimicrobial testing.

2.10. Statistical analysis

At least three parallel tests were performed for all experiments mentioned above. The mean value (x) and the standard deviation (SD) were calculated for each of the following parameters and standard deviations are expressed by error bars in the figures.

3. Results and discussion

3.1. Selected conditions of enzyme immobilization

The capability of immobilization of glucose oxidase and lysozyme onto the polyamide or ionomer film and activity of immobilized enzymes were evaluated in the first part of the study. Polyamide and ionomer films were chosen for the immobilization because these polymers are commonly used for food packaging and they are able to provide free functional groups in their structures after surface activation necessary for binding of enzymes. The formation of covalent bonds between enzyme and polymer was realized *via* Ugi four-component reaction [28,29], which led to stronger bonding of the enzyme, in contrast to the direct covalent bond formed by the reaction of amino groups with glutaraldehyde alone [28]. Different amounts of glucose oxidase and lysozyme were used; the concentration ratio between glutaraldehyde and cyclohexyl isocyanide was established in previous works [28,30]. The surface of films was activated with hydrochloric acid (3 mol L^{-1}) for 20 minutes. This concentration was chosen because the higher concentration of hydrochloric acid caused either rapid degradation of polymer films or less effective surface modification in case of shorter activation time. We found that the activity of immobilized enzyme was nearly 20-times lower after application hydrochloric acid (6 mol L^{-1}) for 2 seconds than hydrochloric acid (3 mol L^{-1}) for 20 minutes because of apparent damage of the films.

3.2. FTIR analysis

FTIR spectroscopy confirmed that both lysozyme and glucose oxidase were covalently bound onto the

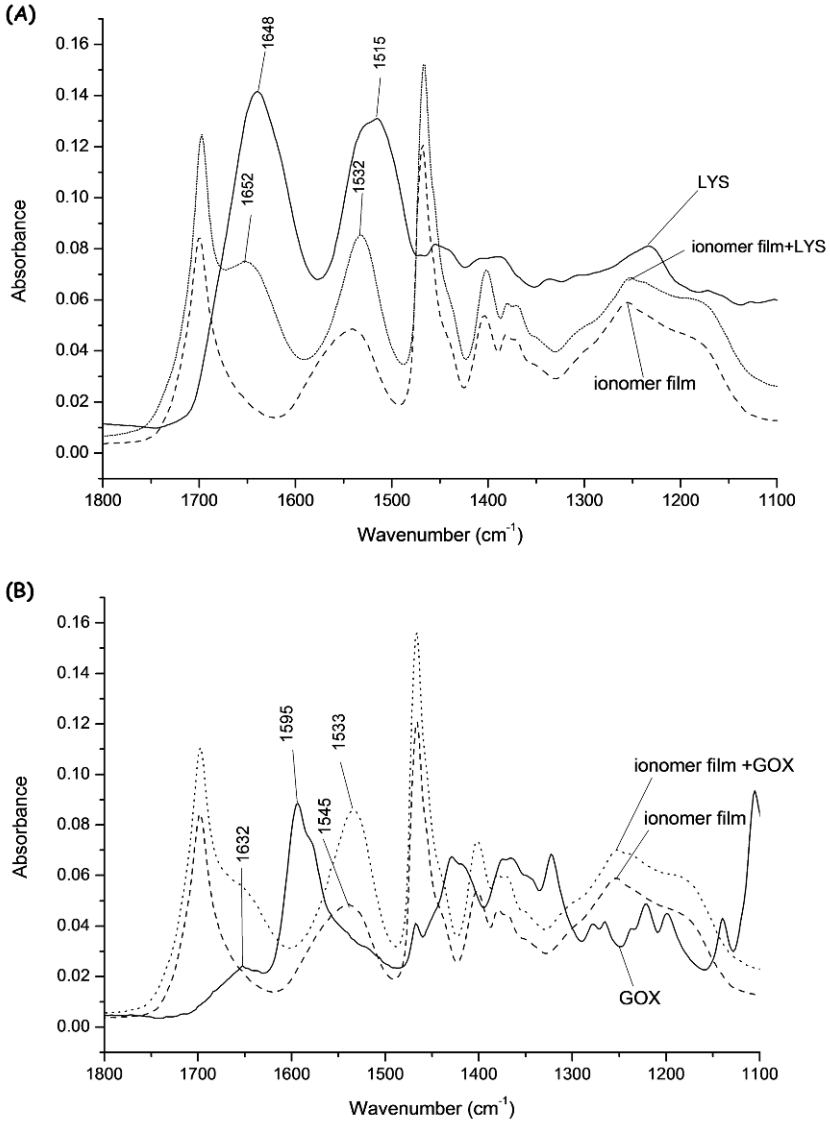

Figure 1. FTIR spectra of ionomer film with immobilized lysozyme (A) and glucose oxidase (B).

surface of polymer films. It is obvious in the comparison of spectra of the films with immobilized enzymes to the spectra of free enzymes. Bands characteristic for the peptidic structure of enzyme, so called Amid I and Amid II, appeared in the spectra of the ionomer film with immobilized enzyme. However, the formation of a covalent bond between the enzyme and film was primarily indicated by the shift of these bands. The positions of the bands of free lysozyme at 1648 cm^{-1} for Amid I and 1515 cm^{-1} for Amid II changed to 1652 cm^{-1} and 1532 cm^{-1} for immobilized enzyme, respectively (Fig. 1A). For glucose oxidase, a significant difference was observed for the band of Amid II, *i.e.*, the shift from 1595 cm^{-1} for the free enzyme to 1533 cm^{-1} for the enzyme bound onto the ionomer film (Fig. 1B). The intensity of Amid I band in the spectra was too low for the assessment of band shift.

Similarly, changes in the spectra of the films with immobilized enzymes to the spectra of free enzymes were also observed for polyamide film. However, the differences in the spectra were minor due to the native structure of polyamide. The small peak shoulder at the main peak of Amid I and Amid II (at 1660 and 1570 cm^{-1}, respectively) is apparent in the spectra of the polyamide film with immobilized LYS and GOX (Fig. 2) indicating modifications in the surrounding of peptidic bond. Additionaly, a new peak at 1480 cm^{-1} belonging to the newly formed terminal $–CH_3$ group was also observed. The films were thoroughly washed after the immobilization procedure to remove residue

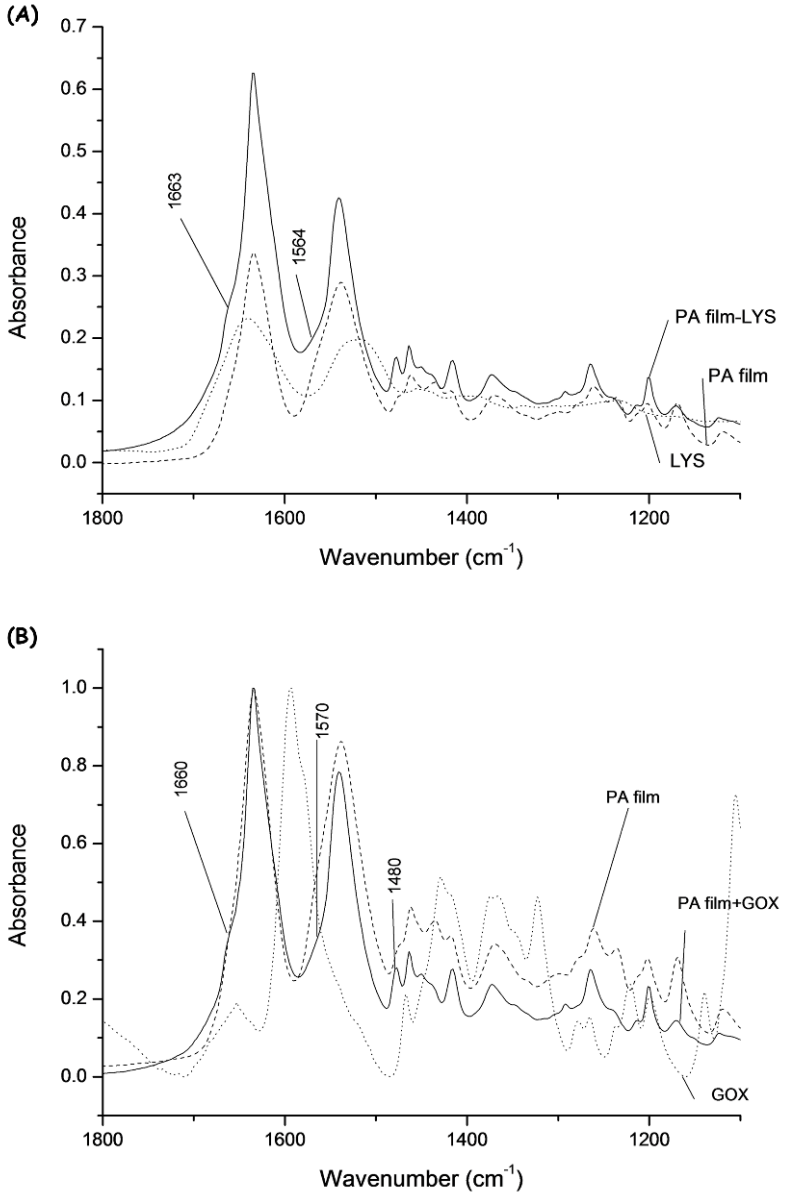

Figure 2. FTIR spectra of polyamide film with immobilized lysozyme (A) and glucose oxidase (B).

of the reaction mixture, therefore the changes in the spectra of the films treated with enzyme solutions can be ascribed to the enzymes directly bound onto the polymer surface.

3.3. Specific activity of immobilized enzymes

The prepared polymer materials were characterized by the specific activity of glucose oxidase and lysozyme measured immediately after the immobilization. The activities of immobilized GOX onto polyamide and ionomer films after incubation at 4°C for 168 hours are given in Table 1. The results indicate that efficiency of immobilization decreased with the increasing amount of the enzyme applied on the polyamide film, although the activity of immobilized GOX increased. Only 10.0%, 1.3% and 0.39% of glucose oxidase was bound onto the polymer surface after application of the enzyme in amount of 0.248, 2.48 and 24.8 U cm^{-2}, respectively. The results were similar in the case of ionomer film. The efficiency of GOX immobilization was 6.6%, 1.6% and 0.25% after applied amount of enzyme of 0.248, 2.48 and 24.8 U cm^{-2}, respectively. The activity of immobilized enzyme did not increase directly proportional to the amount of GOX applied onto the film surface. This phenomenon is common in the case of covalent binding of enzymes. The lower immobilization efficiency could

Table 1. Activity of GOX after immobilization on polyamide or ionomer film and during migration tests.

Amount of enzyme applied on the surface of film (U cm^{-2})	Polyamide film with immobilized glucose oxidase: Activity of immobilized enzyme after incubation for 168 hours (mU cm^{-2})	Activity of migrating enzyme after 48 hours (mU cm^{-2})	Activity of migrating enzyme after 168 hours (mU cm^{-2})			Retained activity of film with immobilized enzyme after migration for 168 hours (mU cm^{-2})		
		pH = 7.0	pH = 2.5	pH = 4.5	pH = 7.0	pH = 2.5	pH = 4.5	pH = 7.0
0.248	24.9 ± 1.9	0.6 ± 0.4	-	-	0.6 ± 0.2	-	-	17.2 ± 2.7
2.48	32.2 ± 0.7	1.8 ± 0.7	0.4 ± 0.2	1.1 ± 0.5	2.2 ± 0.9	5.6 ± 0.2	16.2 ± 0.3	27.1 ± 0.6
24.8	97.7 ± 6.2	6.9 ± 0.5	1.1 ± 0.1	3.7 ± 0.4	7.7 ± 0.3	30.3 ± 0.1	54.9 ± 0.2	79.1 ± 0.5
Amount of enzyme applied on the surface of film (U cm^{-2})	Ionomer film with immobilized glucose oxidase: Activity of immobilized enzyme after incubation for 168 hours (mU cm^{-2})	Activity of migrating enzyme after 48 hours (mU cm^{-2})	Activity of migrating enzyme after 168 hours (mU cm^{-2})			Retained activity of film with immobilized enzyme after migration for 168 hours (mU cm^{-2})		
		pH = 7.0	pH = 2.5	pH = 4.5	pH = 7.0	pH = 2.5	pH = 4.5	pH = 7.0
0.248	16.3 ± 2.2	3.0 ± 0.7	-	-	1.7 ± 0.5	-	-	10.4 ± 1.1
2.48	40.2 ± 3.6	11.4 ± 1.8	6.3 ± 1.6	7.6 ± 1.5	10.9 ± 3.2	3.5 ± 0.3	4.8 ± 0.2	11.1 ± 1.7
24.8	61.8 ± 1.0	28.6 ± 2.2	16.1 ± 0.6	21.1 ± 0.5	29.0 ± 0.8	9.2 ± 0.2	19.9 ± 0.5	24.7 ± 0.3

be caused by many factors such as reaction conditions during immobilization, unfavourable interaction of enzyme with carrier, non-compatible microenvironment and effect of matrice properties especially limited capacity of free binding sites on the polymer surface or hydrophobic character of the surface [31]. The immobilization can, likewise, influence the enzyme structure, mobility and conformational flexibility and last but not least accessibility of the active site of enzyme to substrate due to spatial hindrance [32,33].

The activities of the immobilized GOX depended on the immobilization time. The optimal time for immobilization was found to be 5 days for polyamide film and 7 days for ionomer film. The specific activities of immobilized GOX were lower with shorter or longer times of incubation at 4°C (Table 2).

Lysozyme immobilized onto polyamide and ionomer films after incubation at 4°C for 168 hours was almost inactive, although LYS binding onto the surface of polymer films after the application of all selected enzyme concentrations was confirmed by FTIR spectroscopy. The highest activity of immobilized LYS was 5.7 ± 0.9 U cm^{-2} in the case of polyamide film after applied amount of enzyme of 40000 U cm^{-2} and the activity of LYS was seldom found onto the surface of ionomer film. Both the polymer films did not show enzyme activity after the application of lower amounts of enzyme.

For comparison lysozyme was immobilized onto the polymer films by means of glutaraldehyde in the absence of cyclohexyl isocyanide. The activity of LYS immobilized without application of cyclohexyl isocyanide was comparable to the LYS activity after immobilization with this chemical agent and applied amount of enzyme of 40000 U cm^{-2}. The activities of enzyme immobilized without cyclohexyl isocyanide during incubation time are given in Table 2.

Factors affecting the activity of immobilized enzyme mentioned above can lead to the almost complete loss of activity of immobilized LYS. Appendini and Hotchkiss [7] reported that low activity of LYS immobilized onto activated nylon pellets could have been caused by their small surface area. Additionally, glutaraldehyde that did not react with the support could have induced the loss of activity by crosslinking of the enzyme itself through its amino groups.

3.4. Release of glucose oxidase from films into model solution with various pH

The stability of a polymer-enzyme bond can be influenced by environment. Therefore the migration of GOX from the polymers was assessed. The release of GOX into model solutions with various pH values at 23°C is evaluated in Table 1. The results indicate that 2.3 ± 0.8% of active GOX was released from the polyamide film treated with 0.248 U cm^{-2} of enzyme into the potassium phosphate (0.1 mol L^{-1}) buffer with pH = 7 after migration for 168 hours. The levels of migration were higher for the application of enzyme in the amount of 2.48 and 24.8 U cm^{-2}, *i.e.*, 6.9±2.9% and 7.9±0.3% of the released active enzyme, respectively. The values of migration after 168 hours were comparable to levels of migration after 48 hours. In the case of ionomer film the amount of released active GOX increased from 10.5% to 46.9% with the increasing amount of applied enzyme.

Table 2. Activity of GOX and LYS immobilized on polyamide or ionomer film treated with the amount of 24.8 U cm^{-2} of GOX and 40000 U cm^{-2} of LYS after various incubation time.

Incubation time (day)	Activity of GOX immobilized on polyamide film (mU cm^{-2})	Activity of GOX immobilized on ionomer film (mU cm^{-2})	Activity of LYS immobilized on polyamide film (U cm^{-2})	Activity of LYS immobilized on ionomer film (U cm^{-2})
2	83.4 ± 9.2	42.8 ± 7.2	2.7 ± 0.5	1.5 ± 0.5
5	101.5 ± 9.4	51.6 ± 3.5	4.7 ± 0.3	2.6 ± 0.4
7	59.9 ± 5.9	74.0 ± 5.1	4.8 ± 0.2	2.4 ± 0.3
10	55.3 ± 0.7	47.2 ± 0.6	4.5 ± 0.3	2.3 ± 0.1

The levels of active enzyme were lower in the solutions with pH = 2.5 and 4.5 after migration for 168 hours. The activity of GOX released from the polyamide film into acetic acid (3% w/v) with pH = 2.5 was between 1.0 - 1.5% of the initial film activity for applied amounts of enzyme of 2.48 and 24.8 U cm^{-2}. The results were different for ionomer film. The activity of released enzyme at pH = 2.5 was up to 16% and 26% of the initial activity after the application of enzyme in the amount of 2.48 and 24.8 U cm^{-2}, respectively. These values were lower than those at pH = 7, however, the activity of released enzyme from the ionomer film into solutions with low pH was higher than the retained activity of immobilized GOX after migration described in the next section.

Considering the reactivity of bonds formed during immobilization, the migration of enzyme could be caused by cleavage of the peptide linker. As suggested in Fig. 3A, GOX should be more strongly bound onto the polyamide film than onto ionomer film, due to insertion of the bound enzyme into the polymer chains on the surface of the film. The ionomer film provides one carboxylic group per binding of two enzyme units by Ugi reaction (Fig. 3B). Working on the assumption that the sterical effect of two enzyme molecules in the vicinity of the polymer surface connected with the tension of bonds can play a role, bound complex with GOX could be more readily susceptible to hydrolysis and subsequent release from ionomer film.

The hydrolysis could be further accelerated by lowering the pH because of the higher rate of the peptide bond protonation. This assumption is supported by our observation that with lower pH values the amount of released enzyme increased.

3.5. Activity retention of immobilized glucose oxidase

The character of packaged food can influence the activity of immobilized enzyme, therefore the changes in enzyme activity were observed during the migration test at various pH. Glucose oxidase immobilized onto polymer films maintained activity during migration test for 168 hours (Table 1). Activity retention after migration tests was considerably higher onto the polyamide film. However, it was found that the sum of retained activity of immobilized enzyme and activity of released enzyme after 168 hours was lower than the initial activity of immobilized enzyme before the migration test into the potassium phosphate buffer (0.1 mol L^{-1}) at pH = 7. The total loss of activity ranged between 10-30% and 13-44% in case of polyamide and ionomer film, respectively, depending on initial activity of enzyme. Free enzyme lost 22% of initial activity at pH = 7 during the migration test. The decrease in activity could be caused by inactivation of enzyme; nevertheless it is not evident if the enzyme is inactivated in immobilized form, during cleavage of enzyme from polymer or in solution after release.

The retained activity decreased with decreasing pH of solution applied during migration tests (Table 1). The activity of immobilized GOX was 17.3±0.4% and 31.0±0.1% of the initial activity of immobilized enzyme onto the polyamide film and the applied amount of enzyme of 2.48 and 24.8 U cm^{-2}, respectively, after migration into solution with pH = 2.5. The activity retention of immobilized GOX on the ionomer film was 8.6±1.6% and 14.9±0.3% of initial activity after the application of enzyme in the amount of 2.48 and 24.8 U cm^{-2}, respectively. Whereas activity of free enzyme was not found after 168 hours at pH = 2.5. As mentioned above, the retained activity of immobilized GOX onto the ionomer film was lower than the activity of released enzyme at pH = 2.5 and 4.5.

Although activity of immobilized GOX was partially lost during migration tests, it was observed that immobilized GOX was less affected by pH than the free enzyme. The results indicate that the loss of activity of immobilized and retained enzyme could be caused by enzyme denaturation at low pH. The higher activity of released GOX than the retained activity in case of ionomer film at lower pH could be result of gradual release and simultaneous denaturation of retained as well as released enzyme.

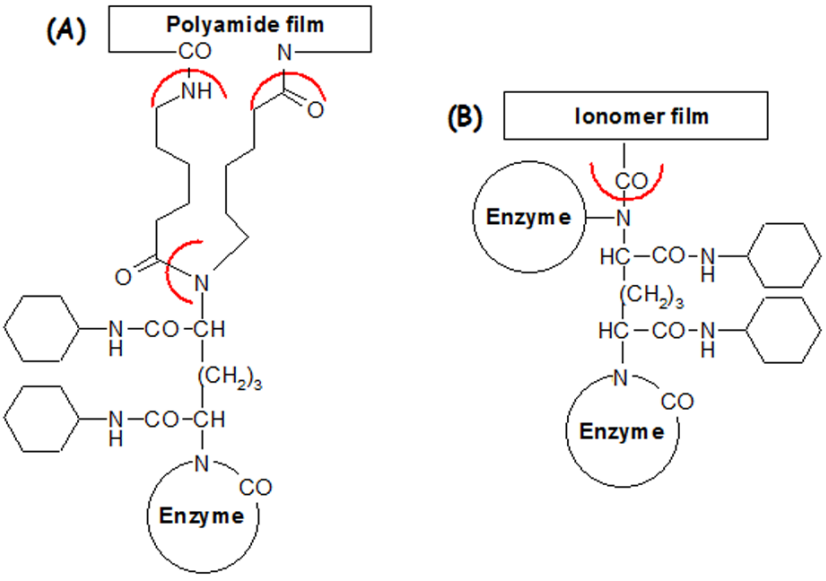

Figure 3. The scheme of linkage between enzyme and polyamide film (A) and ionomer film (B) with indication of hypothetical cleavage (dashed curve) of enzyme from polymer.

3.6. Effect of temperature on activity of immobilized glucose oxidase

The thermal stability of immobilized enzymes is an important requirement for their practical applications as food packaging materials considering the sensitivity of enzymes to higher temperatures commonly applied during food processing. The activity of GOX immobilized on the polyamide film was approximately by 30% higher than the activity of free enzyme in the course of heating at 40, 50 and 60°C for 5 hours (Fig. 4). The activity of GOX immobilized on ionomer film decreased rapidly in the first hour and was lower than the activity of free enzyme in the course of heating at 40 and 50°C for 5 hours. The difference in activity of immobilized GOX and free enzyme was also more significant at 40°C than at 50°C. The lower loss (higher retention) of activity of free GOX than immobilized enzyme in the temperature range of 10-40°C was observed by Ozyilmaz *et al.* [11]. The activity of free GOX was not found after heating at 60°C for 5 hours whereas GOX immobilized on polyamide film and ionomer film maintained at least 36.0±1.6% and 5.6±0.2% of initial activity, respectively. The higher thermal stability of immobilized GOX than free enzyme at 60°C was also reported by Sisak *et al.* [34] and Ozyilmaz *et al.* [11].

3.7. Stability of immobilized glucose oxidase during storage

Certain foodstuffs are often stored for long periods, therefore antimicrobial films should be effective and maintain activity for the whole time of storage. The activity of GOX immobilized on both films moistened with potassium phosphate buffer (0.1 mol L^{-1}, pH 7.0) decreased gradually during storage at 4°C (Table 3). The GOX immobilized on the polyamide film retained more than 90% of its initial specific activity for 40 days of storage, whereas the activity of GOX on the ionomer film was higher than 90% only for 20 days. The immobilized GOX on both films was nearly inactive (≤5%) after 250 days of storage. The obtained results were similar to those of Vartiainen *et al.* [9] and Ozyilmaz *et al.* [11]. They described that activity of immobilized glucose oxidase remained relatively stable during cold storage of films.

3.8. Effect of films with immobilized enzymes on growth of microorganisms

The antimicrobial activity of the prepared films with immobilized enzymes was assessed *in vitro* against selected Gram-positive and Gram-negative indicator bacteria. The polyamide and ionomer films with activity of immobilized glucose oxidase at the levels 30 - 100 mU cm^{-2} inhibited the growth of bacteria *Escherichia coli* CNCTC 6859, *Pseudomonas fluorescens* CNCTC 5793, *Lactobacillus helveticus* CH-1, *Listeria ivanovii* CCM 5884 and *Listeria innocua* CCM 4030 inoculated on agar plates with microorganism concentration of 10^2 – 10^3 CFU mL^{-1} of agar. The visible inhibition zones were formed around the test samples (Fig. 5) and their sizes are given in Table 4. These materials did not affect the growth of *Bacillus subtilis* CNCTC 5615 which could be caused by strong catalase

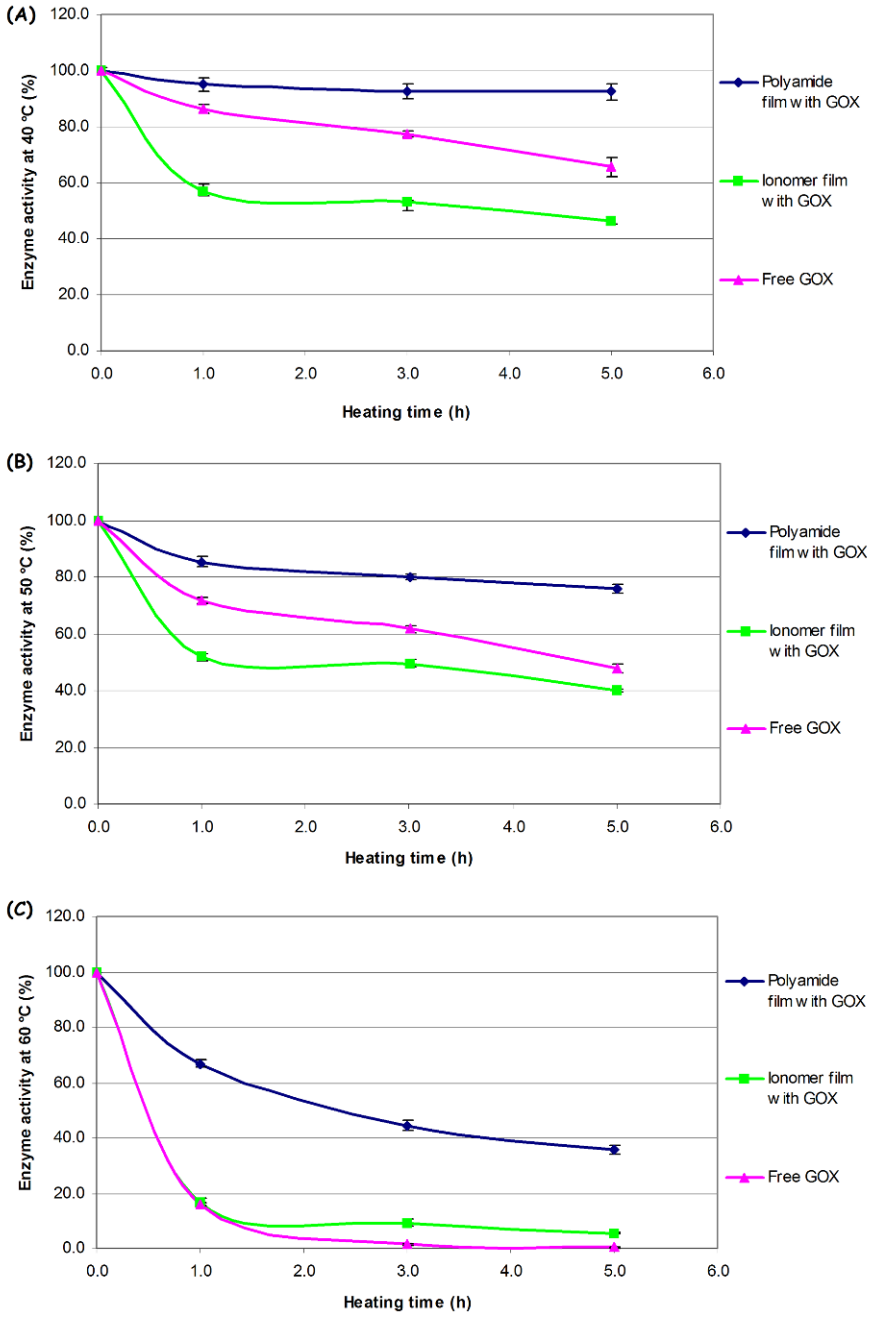

Figure 4. Activity of immobilized and free GOX during heating at 40°C (A), 50°C (B) and 60°C (C).

activity of *Bacillus subtilis* ensuring complete protection of its cells against oxidative stress [35]. The complete inhibition of *E.coli* by GOX covalently immobilized onto biaxially oriented polypropylene (BOPP) films was also reported by Vartiainen *et al.* [9], whereas these films reduced substantially the growth of *B. subtilis*.

The pH values of agar media were lower in the place of contact with film with immobilized GOX after microorganisms incubation due to the formation of gluconic acid, which may also influence the growth of microorganisms [21,26]. The pH of media decreased from 6.3 to 5.1 in case of inhibition growth of *E. coli*, from 6.7 to 5.5 concerning *L. innocua* and only from 6.8 to 6.5 for *B. subtilis*.

The cellulose films with absorbed GOX were prepared for comparison of antimicrobial activity of immobilized and free enzyme. Cellulose film served only as supporting carrier of free enzyme, which was

confirmed by migration tests after that the cellulose film had enzyme activity lower than 1% of initial enzyme activity absorbed on the film. Cellulose film with glucose oxidase (average activity 164±12 mU cm^{-2}) inhibited the growth of the same bacteria as the films with immobilized GOX with difference of size of inhibition zones (Table 4).

The polymer films with immobilized caseinate were also tested to exlcude a potential effect of reagents applied during the immobilization. These films did not inhibit the growth of bacteria mentioned above. Therefore the antimicrobial activity of films can be attributed to the immobilized enzyme.

Table 3. Activity of GOX during the storage of prepared films at temperature of 4°C.

Storage time (day)	Activity of immobilized GOX (%)	
	Polyamide film	Ionomer film
0	100	100
14	98.3 ± 1.4	97.5 ± 1.1
21	97.1 ± 1.7	95.2 ± 1.9
28	93.6 ± 1.4	80.9 ± 1.8
41	92.1 ± 2.3	77.6 ± 2.0
56	73.8 ± 2.2	60.4 ± 1.3
193	17.5 ± 4.2	15.0 ± 2.5
245	3.1 ± 1.4	5.4 ± 1.6

The activity of immobilized GOX onto films was lower after the repeated use during inhibition test. The reused polyamide and ionomer films maintained activity of 6.2 ± 1.5 mU cm^{-2} and 17.9 ± 2.8 mU cm^{-2}, respectively. These films were again capable to inhibit the growth of *E.coli.* The formed inhibition zones were not extensive, *i.e.*, 1.9±0.3 mm for polyamide film and 3.1±0.2 mm for ionomer film. The immobilized GOX was not inactivated by generation of H_2O_2 during test and lowering of pH due to formation of gluconic acid as described [11,26,36].

The antimicrobial activity of films with immobilized GOX was also proven against psychrophilic bacteria *L. ivanovii* (Fig. 5E) and *L. innocua* during cold storage at 4°C for 3 months. The size of inhibition zones was 9.8±0.5 mm for *L. innocua* and 13.6±0.8 mm for *L. ivanovii* around the polyamide film and 7.9±0.7 mm for *L. innocua* and 8.8±0.9 mm for *L. ivanovii* in the case of ionomer film.

None of the polyamide and ionomer films with immobilized lysozyme was capable of inhibiting the indicator bacteria. However, cellulose film with absorbed LYS (average activity 1300 ± 140 U cm^{-2}) substantially inhibited the growth of Gram-positive bacteria *Lactobacillus helveticus* CH-1 and *Bacillus subtilis* CNCTC 5615 (Fig. 5F) presumably due to the

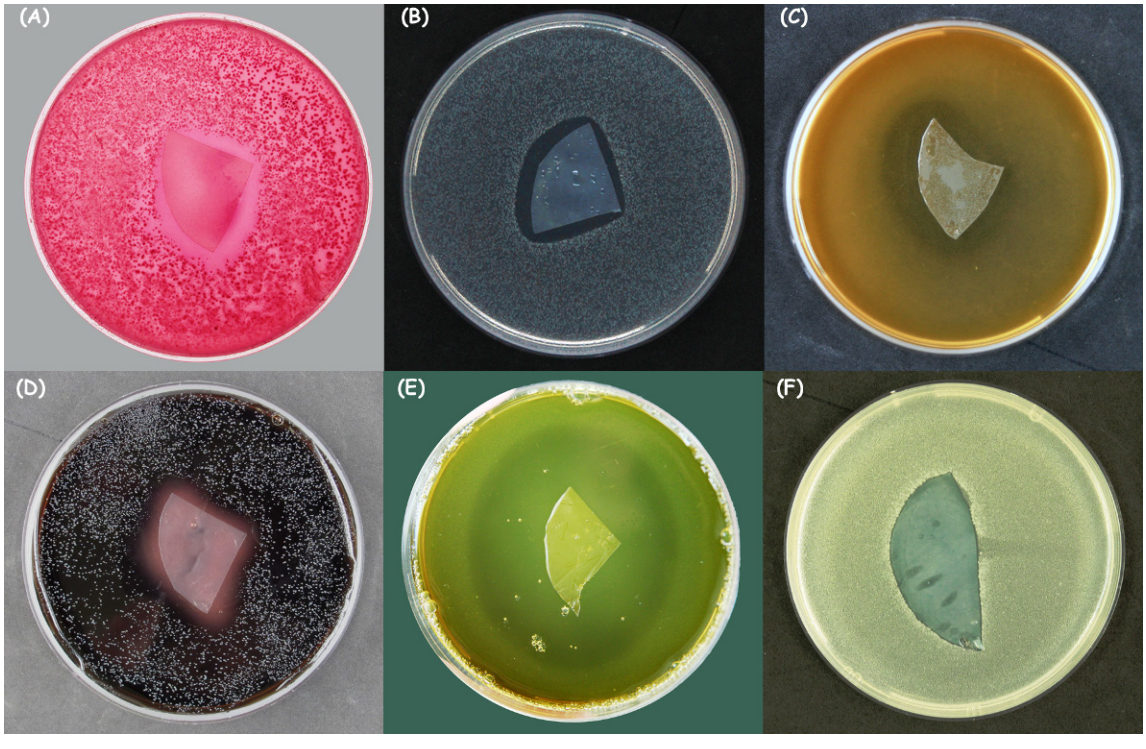

Figure 5. The growth inhibition of indicator bacteria: *Escherichia coli* (A) and *Pseudomonas fluorescens* (B) by ionomer film with GOX; *Lactobacillus helveticus* (C) and *Listeria innocua* (D) by polyamide film with GOX; *Listeria ivanovii* (E) by polyamide film with GOX during cold storage at 4°C; *Bacillus subtilis* (F) by cellulose film releasing LYS.

Table 4. Size of inhibition zones formed around the films with immobilized GOX.

Bacteria	Size of inhibition zone (mm)		
	Polyamide film	Ionomer film	Cellulose film
***Escherichia coli* CNCTC 6859**	5.2 ± 0.6	4.3 ± 0.3	8.7 ± 0.5
***Pseudomonas fluorescens* CNCTC 5793**	2.8 ± 0.3	4.4 ± 0.2	8.2 ± 0.5
***Lactobacillus helveticus* CH-1**	6.0 ± 1.1	5.1 ± 0.4	10.9 ± 0.9
***Bacillus subtilis* CNCTC 5615**	x	x	x
***Listeria ivanovii* CCM 5884**	2.6 ± 0.7	3.7 ± 0.5	5.6 ± 0.4
***Listeria innocua* CCM 4030**	3.8 ± 0.8	4.0 ± 0.1	6.1 ± 0.3

action of released free enzyme. However, it is necessary to consider that LYS is most active on certain Gram-positive bacteria, its antimicrobial activity can vary widely among groups of similar microorganisms and its lytic activity depends on many other parameters [24]. The antimicrobial packaging materials with completely immobilized LYS have already been prepared and described by Conte *et al.* [4] and Appendini *et al.* [7], however their antimicrobial activities were especially tested against *M. lysodeikticus*, which is particularly sensitive to LYS [7,24]. Lysozyme showed antimicrobial effect on *Bacillus subtilis* and *Lactobacillus plantarum* provided that it released from the films with incorporated enzyme at the levels of 700 – 1400 U cm^{-2} [37].

4. Conclusions

Our results demonstrate that the application of Ugi reaction is suitable to immobilize glucose oxidase on commonly used and relatively inert polymer films producing enzymatically active materials. However, immobilization of lysozyme via covalent attachment seems to be unsuitable due to substantial loss of enzyme activity. The activity of immobilized glucose oxidase increased with increasing amount of applied enzyme on the film surface, although not directly proportional. Release of glucose oxidase from the polyamide film into model solutions was lower than 10% of initial activity retaining up to 80% of activity of immobilized enzyme after the migration tests at neutral pH. Immobilized glucose oxidase has higher storage and thermal stability and it is less sensitive to pH changes than free enzyme.

The prepared packaging materials with immobilized glucose oxidase have sufficient activity to inhibit the growth of selected bacteria on the agar plates. Therefore further research should be focused on the practical application of these materials in food packaging, especially on the testing of their antimicrobial effect on microorganisms of concern in packaged foodstuffs during storage.

Acknowledgements

The authors are grateful for financial support from specific university research (MSMT No 21/2012) and research project (MSMT No. 6046137305).

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