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EFFECT OF PULLULAN COATING ON INHIBITION OF CHOSEN MICROORGANISMS' GROWTH

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Background. Pullulan is a water-soluble polysaccharide produced by fungi *Aureobasidium pullulans*. This glucan was applied for coating of food products. The aim of this study was obtaining of a pullulan coating and checking its effect on growth of microorganisms responsible for spoilage of food.
Materials and methods. Pullulan produced by the white mutant *A. pullulans* B-1 was applied. Permeability of oxygen and carbon dioxide through film produced from 10% water solution of pullulan was checked as well as degree of inhibition of chosen microorganisms through pullulan coating formed on surface of growth's media.
Results. Low permeability of gases through pullulan film and a considerable growth's limitation of all tested microorganisms were found. A total growth's inhibition of 21 from 36 tested strains and a partial growth's limitation of the remaining 15 strains was observed. The inhibitory effect was diverse and it was from 63 to 100%.
Conclusions. These results proved that pullulan coating applied in these tests revealed big barrier characteristics in relation to oxygen and carbon dioxide, which had effect upon growth's inhibition of most of the tested microorganisms, responsible for spoilage of food.

Key words: edible coating, pullulan, microbiological contamination of food

INTRODUCTION

Progressing social-economic development, increasing requirements of consumers as well as bigger and bigger demand on portioned and processed to a minimum degree food [Cichoń and Miśniakiewicz 2000], functional and convenience [Czapski 2002], intention to diversify and to make product more attractive, on the other hand to prolong offered product's durability time, it requires from food manufacturers not only changes in technological processes, but also looking for new methods of food packing. Into such

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methods coating of raw materials and food products with edible coatings can be included [Debeaufort et al. 1998]. Safe for human health and for environment, produced from biodegradable materials they are an alternative for plastic packagings.

The main purpose of food's packing is limitation of a negative effect, which environment has upon a product and preserving product's high quality during the whole period of its usefulness for consumption. Edible coatings should delay loss of moisture, limit migration of fatty compounds and solved substances, retain volatile components and be a carrier of supplementary substances [Krochta and De Mulder-Johnston 1997, Petersen et al. 1999, Tharanathan 2003]. Basic components for producing of edible coatings are fats, proteins and polysaccharides [Tharanathan 2003]. Coatings formed from lipids, owing to their hydrophobic characteristics are a good barrier for water vapour, inhibiting its evaporating out of product, but they are characterised by low mechanic properties and high permeability of oxygen [Kester and Fennema 1989]. Then coatings produced of proteins or polysaccharides have good mechanic characteristics and at low humidity of environment they inhibit access of oxygen to product, still they are generally hydrophilic, they easily absorb water and are characterised by high permeability of water vapour [Li and Chen 2000, Gontard et al. 1996]. In order to improve characteristics of produced films and edible coatings instead of one component a mixture of several substances is applied [Guilbert et al. 1996, Diab et al. 2001, Petersen et al. 1999]. Most often hydrocolloids are combined (proteins, polysaccharides), which form a crosslinked, integral and coherent matrix with hydrophobic compounds (lipids), which mainly improve barrier characteristics of film in relation to water vapour. Shih [1996] found, that film formed from rice protein and pullulan mixed in the ratio of 1:1 was characterised by better mechanic properties (among others it had higher tear resistance) in relation to films made from single components.

Pullulan is a polysaccharide soluble in water, produced by fungi *Aureobasidium pullulans* [Yuen 1974, Simon et al. 1995]. It is not toxic for people and animals. It is low-caloric and totally biodegradable [Leathers 2003, Lee et al. 2001]. Pullulan reveals good adhesive characteristics, that enables it to be applied for coating of food products. Coatings made of pullulan are colourless, odourless and have no taste. They can be very thin, even of thickness of about 0.01 mm [Yuen 1974]. Pullulan coatings can be applied for prolongation of fruit and vegetables' durability. Coating formed on strawberries and kiwi fruit had an advantageous effect upon maintaining of firmness, colour and upon reduction of fruit weight's decrease [Diab et al. 2001]. A similar effect was obtained applying to kiwi fruit a coating from a mixture of pullulan, soya protein, glycerol and stearic acid. The coated fruit stored at ambient temperature underwent softening process three times more slowly than uncoated fruit [Xu et al. 2001].

There are no direct data concerning possibility of inhibition of micro bacterial growth by pullulan coatings. That is why the purpose of this work was obtaining of a pullulan coating and checking in model tests its effect on growth of microorganisms, which are the reason of food's decay.

MATERIALS AND METHODS

Biological material, conditions of culture and production of pullulan

In the tests a white mutant of the fungus *Aureobasidium pullulans* B-1 was used, which had been selected after an associated mutagenisation (UV and ethyleneimine) of a wild strain *A. pullulans* A.p.-3. Mutant B-1 in comparison with the natural parental strain is characterised by higher production of pullulan and by lack of synthesis of melanin compounds, contaminating raw pullulan preparation [Gniewosz et al. 1999, Gniewosz and Duszkiewicz-Reinhard 2008]. The strain was cultivated on substrate of the following composition (g/L): saccharose 60, K_2HPO_4 7.5, NaCl 1.5, $(NH_4)_2$ SO_4 0.72, $MgSO_4 \cdot 7H_2O$ 0.4, yeast extract 0.4, pH was 6.0 ±0.5 [Gniewosz et al. 1997]. The culture was cultivated at the temperature of 28°C for 48 hours in a SM-30/C shaker, (Otto Gmbh, Germany) of 200 rpm. Next 1 mL inoculum was transferred into fresh liquid substrate and culture of the fungus was carried out in the same conditions as previously (28°C, 200 rpm) for 96 hours. After that time of culture biomass of the fungus was centrifuged at 18 000 x g for 20 minutes (Centrifuge 5804 R, Eppendorf, Germany). Next pullulan was precipitated from the post-culture liquid by use of 96% ethanol, which was added to supernatant in the ratio of 1:1 (v/v). The precipitated pullulan was centrifuged at 11 000 x g for 10 minutes, and then it was subjected to purification after the method of Roukas and Biliaderis [1995]. Pullulan was dried at the temperature of 55°C and ground in a mill.

Determination of oxygen and carbon dioxide's permeability through pullulan film

The film was obtained from 10% water solution of pullulan, which in quantities of 10, 15 and 20 mL was poured onto sterile Petri plates of 15 cm diameter. In order to dry partly the films the plates were placed in a laminar chamber under blow of sterile air for 2 hours. Next the plates were left until the film was completely dried at room temperature (22°C) and at a relative humidity of air 55%. Next the films were carefully taken off the glass surface and carried over into a chamber of constant relative humidity of air (RH 50%) and at the temperature of 22°C for three days. After the films had been dried, they were measured by means of 122 DM apparatus (Mercer) and fragments were cut out of the thickness respectively 15 ±3, 25 ±5 and 40 ±5 μm.

Determination of oxygen's permeability of films prepared in such a way was carried out in accordance with ASTM D 3985-05 norm by means of OX-TRAN 100 apparatus with a coulometric detector. As gas carrier nitrogen containing 3% hydrogen was applied. The test was carried out at the temperature of 23 ±2°C and at relative humidity 0%. Determination of carbon dioxide's permeability was carried out by means of Permatran C-200 (Mocon, USA) with a sensor in infrared range (wave length 4.3 μm). As gas carrier dry nitrogen was used. The test was carried out at the temperature of 23°C and at relative humidity from 50 to 1%. Both determinations were carried out in the Central Research and Development Center (COBRO) in Warsaw.

Test strains of fungi and bacteria

In the tests the following pure cultures were used:

- moulds: *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus glaucus*, *Aspergillus niger* ATCC 9142, *Aspergillus oryzae*, *Geotrichum candidum*, *Botritis cinerea* E 92, *Cladosporium herbarum*, *Fusarium avenaceum* F VIII, *Fusarium species* F VII, *Monilia species*, *Mucor mucedo* KKP 461, *Penicillium notatum* E 30, *Penicillium roqueforti* E 31, *Rhizopus arrhizus* ATCC 11145, *Rhizopus nigricans* KKP 484, *Rhizopus oryzae* M/180, *Trichoderma harzianum* KKP 534,
- yeasts: *Candida mycoderma*, *Candida utilis* ATCC 9950, *Endomyces magnusi*, *Hansenula anomala* R 26, *Kluyveromyces fragilis* R 11, *Pichia jadinii*, *Rhodotorula rubra* Rh VIII, Saccharomyces cerevisiae ATCC 2366, *Schizosaccharomyces pombe* R 25, *Torulopsis utilis* R 10,
- bacteria: *Bacillus subtilis* ATCC 6650, *Citrobacter freundii* ATCC 8090, *Escherichia coli* ATCC 25922, *Lactobacillus plantarum* ATCC 4080, *Micrococcus luteus* ATCC 9341, *Pseudomonas fluorescens*, *Sarcina* sp., *Tetracoccus* sp.

All the strains came from the Collection of Pure Cultures of Department of Biotechnology and Food Microbiology of Warsaw University of Life Sciences (SGGW). The strains of fungi were stored on YPD medium, and bacteria strains on Nutrient Agar medium or MRS broth (*Lb. plantarum* ATCC 4080) at the temperature of 4°C.

Methods of carrying out of tests strains' culture under pullulan coating

Inoculum of microorganisms (10^5-10^6 CFU/mL) was carried over on Petri plates and a corresponding medium was poured on them. On dispersion of moulds' spores Sabourand Agar medium (BTL, Poland) was poured, on dispersion of yeasts YPD medium of the composition (g/L): glucose 10, peptone 20, yeast extract 10, agar 25 was poured, and on dispersion of bacteria Nutrient Agar medium or in case of *Lb. plantarum* MRS medium (Merck, Germany) was poured. On surface of each medium 1 mL of 10% water solution of pullulan was carried over. For quicker drying of the pullulan coating the plates were placed for about 1 hour in a laminar chamber with a constant flow of sterile air. In parallel control plates were prepared (uncovered with pullulan coating). After the coating has got set, the plates with the pullulan coating and without it were incubated at the temperature of 28°C or 37°C for 24-96 hours (depending on the strain). After that period of time the grown up colonies of microorganisms were counted. The test was carried out in three series. On the base of the obtained results the ratio of the number of microorganisms' colonies grown up on the plates with the pullulan coating to the number of colonies grown up on the control plates was calculated. Thus obtained results were used for calculation of inhibition degree of the tested bacteria's growth by the pullulan coating.

DISCUSSION AND RESULTS

Testing of permeability degree of oxygen and carbon dioxide through pullulan film

Control of concentration level of oxygen and carbon dioxide around food products is a well known method of prolonging food's durability. Oxygen accelerates irreversible processes of majority of dyestuffs and vitamins' degradation, and it is one of the reasons of unsaturated lipids' auto oxidation as well [Wilska-Jeszka 2002, Drozdowski 2002].

That is why high barrier characteristics of coatings in relation to gases is very much desirable.

The formed pullulan film was subjected to testing of oxygen and carbon dioxide's permeability. Permeability of oxygen was between 570-6100 cm^3/m^2 of film during 24 hours at the pressure of 0.1013 MPa, at the ambient relative humidity of 0% and it was closely connected with film's thickness. The thicker pullulan film, the lower permeability of oxygen was observed (Table 1). At a mean thickness of pullulan film of 25 μm oxygen's permeability was at the level of 1600 cm^3/m^2/24 hours, which testifies to the fact, that pullulan film is a good barrier for oxygen. The obtained results are comparative with oxygen's permeability through polypropylene and polyethylene films, whose permeability of this gas was respectively 1600 cm^3/m^2/24 hours and 1400-8000 cm^3/m^2/24 hours [Janicki and Ćwiek-Ludwicka 2003].

Table 1. Barrier specificity of pullulan film

Kind of research	RH %	Thickness of film μm ±SD	Results of designations $cm^3/m^2 \cdot 24\ h \cdot 0.1$ MPa
Permeability of oxygen	0	15 ±5	6 100
		25 ±5	1 600
		40 ±5	570
Permeability of carbon dioxide	50	25 ±5	< 100
	1		< 10

Literature data make known, that permeability of oxygen and carbon dioxide of various materials depends not only on films' thickness, but on values of environment's relative humidity as well. Gontard and al. [1996] observed, that at low relative humidity value edible film of wheat gluten revealed low permeability of oxygen and carbon dioxide, respectively 1.24 and 7.4 amol/(Pa·m·s). At humidity over 60% permeability of these both gases increased rapidly up to the values of 1290 and 36 700 amol /(Pa·m·s). In the tests carried out in the present work a similar tendency was observed. With drop of relative humidity permeability of coating dropped in relation to carbon dioxide. At coating's thickness of 25 μm and with humidity drop from 50% to 1% reduction of permeability of CO_2 from 100 $cm^3/m^2 \cdot 24$ hours $\cdot$ 0.1 MPa to 10 $cm^3/m^2 \cdot 24$ hours $\cdot$ 0.1 MPa was observed.

Effect of pullulan coating on speed of growth of chosen moulds' strains

On the base of the obtained results for each of the tested moulds' strains degree of growth's inhibition under pullulan coating in relation to growth on control substrate (without any coating) was calculated. The obtained results were presented in the Figure 1.

In case of all the 18 tested moulds' strains inhibitory effect of applied pullulan coating was observed. The degree of growth's inhibition was diverse for various strains and it was from 54% to 100%. 12 tested moulds did not reveal any growth on the substrate

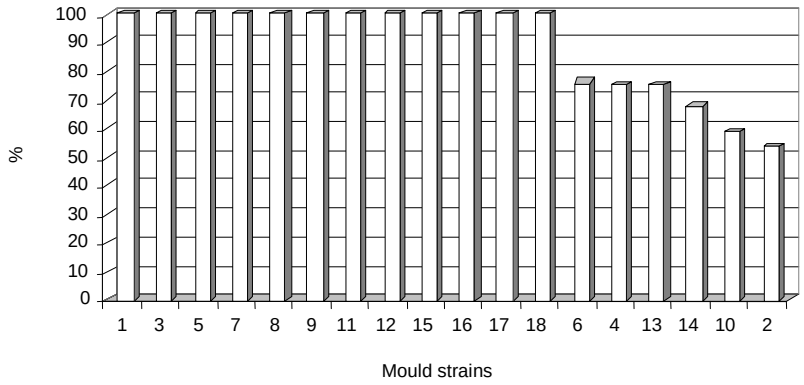

Fig. 1. The degree of growth's inhibition of chosen moulds' strains through pullulan coating: 1 – *Alternaria alternata*, 3 – *Aspergillus glaucus*, 5 – *Aspergillus oryzae*, 7 – *Cladosporium herbarum*, 8 – *Fusarium avenaceum* F VIII, 9 – *Fusarium* sp., 11 – *Monilia* sp., 12 – *Mucor mucedo* KKP 461, 15 – *Rhizopus arrrhizus* ATCC 11145, 16 – *Rhizopus nigricans* KKP 484, 17 – *Rhizopus oryzae* M/180, 18 – *Trichoderma harzianum* KKP 534, 6 – *Botritis cinerea* E 92, 4 – *Aspergillus niger* ATCC 9142, 13 – *Penicillium notatum* E 30, 14 – *Penicillium roqueforti* E 31, 10 – *Galactomyces geotrichum*, 2 – *Aspergillus flavus*

with carried on pullulan coating, which makes two third of all tested strains. In this group there were: *Alternaria alternata*, *Cladosporium herbarum*, *Monilia* sp., *Trichoderema harzianum* KKP 534 and two species of each of the genus *Aspergillus*, *Rhizopus* and *Fusarium.* The least sensitive to presence of the pullulan coating proved to be the strain *Aspergillus flavus*, still its growth under the coating was inhibited in 54% as well.

Xu et al. [2001] testing coatings on base of rice protein with supplement of pullulan found, that presence of this compound in the coating had an advantageous effect upon increasing of the coating's barrier characteristics in relation to oxygen. Similar results were obtained by Yuen [1974] and Roller and Dea [1992], who suggest that the mechanism of pullulan coating's activity resolves itself into a mechanic severance of oxygen's access from environment. Because moulds are organisms that require presence of oxygen for their growth, and the formed in the present tests pullulan coating was characterised by a big ability of limitation of gas exchange between environment and the coated material, this phenomenon was probably the reason of mould's growth inhibition.

In the carried out tests limitation of six other moulds' growth was observed as well. They were: *Botritis cinerea* E 92, *Penicillium notatum* E 30, *Aspergillus niger* ATCC 9142, *Penicillium roqueforti* E 31, *Galactomyces geotrichum* and *Aspergillus flavus.* A significant factor limiting effect of pullulan coating on growth of these moulds was delay of sporification. It was probably brought about by the fact, that colonies of the moulds on plates with the carried on pullulan coating were appearing with 24 hours' delay in relation to growth of the moulds on the control plates.

Zhang and Quantick [1998] testing effect of chitosan coating upon prolongation of fresh raspberries and strawberries' durability period found, that application of this coating limited growth of moulds *Botritis cinerea* and *Rhizopus* sp. The inhibitory effect was contained within the range from 40 to 62%.

Effect of pullulan coating on speed of growth of chosen yeast strains

On base of the obtained results for each of the tested yeast strains degree of growth's inhibition on plates with pullulan coating in relation to growth on control plates was determined. The obtained results were presented in the Figure 2.

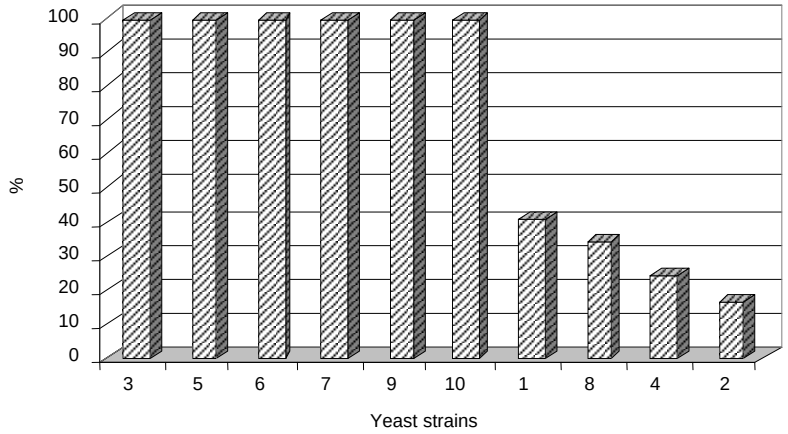

Fig. 2. The degree of growth's inhibition of chosen yeast' strains through pullulan coating: 3 – *Endomyces magnusi*, 5 – *Kluyveromyces fragilis* R11, 6 – *Pichia jadinii*, 7 – *Rhodotorula rubra* Rh VIII, 9 – *Schizosaccharomyces pombe* R25, 10 – *Torulopsis utilis* R10, 1 – *Candida mycoderma*, 8 – *Saccharomyces cerevisiae* ATCC 2366, 4 – *Hansenula anomala* R 26, 2 – *Candida utilis* ATCC 9950

All tested yeast strains revealed a limited growth on substrate with pullulan coating. The inhibitory effect was contained in the range from 14 to 100%. Out of 10 tested yeast strains, in case of six of them total inhibition of growth on substrate coated with pullulan coating was observed. This group implied: *Endomyces magnusi*, *Kluyveromyces fragilis* R 11, *Pichia jadinii*, *Rhodotorula rubra* Rh VIII, *Schizosaccharomyces pombe* R 25 and *Torulopsis utilis* R 10.

In case of the four remaining yeast strains their growth under pullulan coating was slower in relation to the control culture. The least sensitive to presence of pullulan coating proved to be the strain *Candida utilis* ATCC 9950. Its growth was inhibited only in 14%, which may prove, that this species is little sensitive to unfavourable environmental conditions, into which a limited access of oxygen can be numbered.

Effect of pullulan coating upon speed of growth of chosen bacteria strains

Five strains of Gram (+) bacteria and three strains of Gram(-) bacteria were chosen for the tests. Part of them reveal oxygen metabolism, the remaining ones are relative anaerobes. On base of the obtained results for each of the eight tested bacteria strains inhibition's degree of growth on substrate with pullulan coating in relation to the controlled substrate was calculated. The obtained results were presented on the Figure 3.

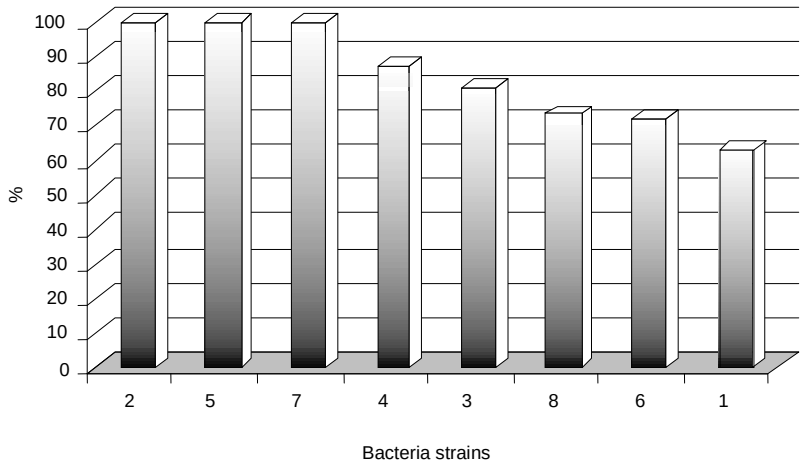

Fig. 3. The degree of growth's inhibition of chosen bacteria' strains through pullulan coating: 2 – *Citrobacter freundii* ATCC 8090, 5 – *Lactobacillus plantarum* ATCC 4080, 7 – *Pseudomonas fluorescens*, 4 – *Lactobacillus brevis*, 3 – *Escherichia coli* ATCC 25922, 8 – *Tetracoccus* sp., 6 – *Micrococcus luteus* ATCC 9341, 1 – *Bacillus subtilis* ATCC 6650

In case of all tested bacteria strains inhibitory effect of the applied pullulan coating was observed. The inhibitory effect was diverse for the tested strains and it was from 63 to 100%. The most limited was growth of the strains *Citrobacter freundii* ATCC 8090, *Lactobacillus plantarum* ATCC 4080 and *Pseudomonas fluorescens.* The least sensitive to presence of pullulan coating proved to be the strain *Bacillus subtilis* ATCC 6650. As it is reported by Burbianka and Pliszka [1983] numerous species of the genus *Bacillus* can also reproduce at reduced oxygen pressure, especially in presence of hydrocarbons.

Kandemir et al. [2005] tested film produced on basis of pullulan with supplement of EDTA·H_2O and lysozyme. They observed growth inhibition of the strain *Escherichia coli* and lack of inhibitory effect on growth of the strain *Lactobacillus plantarum.* Still the results of these tests are not completely comparative with the ones obtained in the present work, because EDTA and lysozyme included into the film are known antibacterial agents. EDTA has lytic effect upon many G (-) bacteria, on the other hand lysozyme is an enzyme which decomposes cell wall of Gram (+) bacteria [Mecitoğlu et al. 2006].

SUMMARY

Pullulan coating applied in these tests revealed big barrier characteristics in relation to oxygen and carbon dioxide, which had effect upon growth's inhibition of most of the tested microorganisms, responsible for decay of food. Its additional favourable advantage is that inhibitory effect on growth of microorganisms was obtained without applying supplementary substances having antimicrobial effect. The results of the carried out tests suggest, that pullulan coating can be an useful element to ensure microbiological safety of food, thus–to prolong food's durability period.

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WPŁYW POWŁOKI PULLULANOWEJ NA HAMOWANIE WZROSTU WYBRANYCH DROBNOUSTROJÓW

Wprowadzenie. Pullulan jest rozpuszczalnym w wodzie polisacharydem wytwarzanym przez grzyby *Aureobasidium pullulans*. Glukan ten znalazł zastosowanie do powlekania produktów żywnościowych. Celem badań było otrzymanie powłoki pullulanowej i sprawdzenie jej wpływu na wzrost drobnoustrojów, będących przyczyną psucia się żywności.
Materiały i metody. Zastosowano pullulan wytwarzany przez białego mutanta *A. pullulans* B-1. Sprawdzano przepuszczalność tlenu i dwutlenku węgla przez film wytworzony z 10-procentowego wodnego roztworu pullulanu oraz stopień zahamowania wybranych drobnoustrojów przez powłokę pullulanową utworzoną na powierzchni podłoża wzrostowego.
Wyniki. Stwierdzono małą przepuszczalność gazów przez film pullulanowy oraz znaczne ograniczenie wzrostu wszystkich badanych drobnoustrojów. Obserwowano całkowite zahamowanie wzrostu 21 z 36 badanych szczepów oraz częściowe ograniczenie wzrostu pozostałych 15 szczepów. Efekt hamujący był zróżnicowany i wynosił od 63 do 100%.
Wnioski. Zastosowana w badaniach powłoka pullulanowa wykazała dużą barierowość w stosunku do tlenu i dwutlenku węgla, co wpłynęło na hamowanie wzrostu większości badanych drobnoustrojów, odpowiedzialnych za psucie się żywności.

Słowa kluczowe: powłoka jadalna, pullulan, mikrobiologiczne zanieczyszczenie żywności

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