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EFFECT OF BIOFILM FORMATION BY *PSEUDOMONAS AERUGINOSA* ON GAS PERMEABILITY OF FOOD WRAPPING FOILS

Elżbieta Bogusławska-Wąs[1], Sławomir Lisiecki[2], Anna Drozdowska[1], Katarzyna Ilczuk[1]

[1]Department of Food Microbiology, [2]Department of Packaging and Biopolymers; Faculty of Food Science and Fisheries, Agricultural University of Szczecin, Szczecin

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The natural precedence of microorganisms growth on the damp surfaces is the formation of biofilm. The arising biolayer, stabilized by extracellular substances, is becoming hard to remove biological structure, the enzymatic activity of which can lead to violation of the packaging, and consequently to acceleration of the process of food spoilage.

The microorganisms of *Pseudomonas* species are widely spread in food products' environment. In order to perform the analysis three types of food wrapping foils from polyamide – polyethylene (PA/PE), which were kept in *Pseudomonas* culture, were used in the study. Two strains were used – the standard ATCC 15442 (WZ) and the strain isolated from pork-beef minced meat (MB) airtight packaged. All cultures were run at the temperatures 4°C and 20°C. It was reported that on all types of food wrapping foils biofilms were developed, which were formed by *Ps. aeruginosa*. The biolayers developed decreased permeability of foils, which was shown by restriction of permeability for gases. The changes of foils properties analysed here were most of all dependent on the type of the foil.

INTRODUCTION

The utilization of foils in the food industry is the standard in food technology nowadays. Proecological trends, together with more strict regulations concerning packaging waste, promote the development of materials safe for environment. Biodegradable food wrapping foils, available on the market, have attributes excluding them from the use as a packages for greasy or frozen food [Bartkowiak *et al*., 2004]. For these products use is made of packaging foils made of artificial materials which protect easily spoilong food. The requirement for their usage is proper binding of different polymers with specified features [Michniewicz, 1999]. Polyamide (PA) and polyethylene (PE) or their derivatives are commonly used materials with supplementing properties. Good mechanical properties, attrition resistance and temperature stability of polyamide, and very low permeability for gases and susceptibility for welding of polyethylene, predispose these synthetic foils (PA/PE) to be exploited in current packaging methods. In vacuum packaging techniques, modified atmosphere packaging (MAP) and controlled atmosphere packaging (CAP), aseptic packaging and with oxygen adsorbents, the proper choice of package has the influence on the final quality and durability of a product [Czerniawski & Stasiek, 2001]. Lowering oxygen contents and increasing the amount of CO_2, as a result of tissue and microbial respiration, limit the growth of facultative anaerobic microorganism, mostly *Pseudomonas*, *Alteromonas* and *Moraxella*, which contributes to the extension of product durability.

An obvious result of growth of microorganisms on damp surfaces is the formation of biofilm. Forming biolayer, stabilized by extracellular substances, becomes very hard to be removed microbiological structure [Gilbert *et al*., 2003], the enzymatic activity of which can lead to violation of package's structure and, consequently, to speed up food spoilage processes. Improper usage of comestible foils as well as inappropriate microbiological quality of packaging product can contribute to the growth of spoilage microflora and pose danger to the consumer.

Taking into consideration the above factors, the aim of this work was to determine the effect of growing bacterial biofilm on the properties of food wrapping foils used.

MATERIALS AND METHODS

Food wrapping foils. In order to carry out the tests three polyamide/polyethylene (PA/PE) food wrapping foils were used (F1, F3, F5), admitted for use in the food industry. The foil F5 additionally contained vinyl-ethylene alcohol (EVOH). All of the foils used were produced with the coembossment method using chill roll. Properties of the foils are given in Table 1.

Author's address for correspondence: Elżbieta Bogusławska-Wąs, Agricultural University of Szczecin, Faculty of Food Science and Fisheries, Department of Food Microbiology, Papieża Pawła VI 3, 71-459 Szczecin, Poland; tel./fax: (48 91) 42 50 407; e-mail: ewas@tz.ar.szczecin.pl

TABLE 1. Properties of foils used in testing.

Foil labeling	Manufacturer labeling	Chemical constitution of foil	Foil thickness (nm)
F1	GPM	PA/PE	90
F3	GPM	PA/PE	40
F5	GPP	EVOH	80

***Ps. aeruginosa* strains.** The standard strain of *Ps. aeruginosa* ATCC 15442 (WZ), and the strain *Ps. aeruginosa* (MB) isolated from hermetically-packaged pork-beef minced meat were used for testing.

Isolation and identification of strains from minced meat. *Pseudomonas sp* strains were isolated from meat according to Polish Norm [PN-85/A-82051]. The cultures were run in three repetitions using the method of surface culture on Pseudomonas Agar base (Oxoid) with CFC Selective Agar Supplement (Oxoid). Plates were incubated for 3 days at room temperature. Blue and green fluorescent colonies were cultured on Nutrient Agar (Oxoid) for further identification.

In order to establish which species the strains from 24-h colony belong to, the Gram staining and oxidase tests were carried out. For further identification G(-) and oxidase(+), bacteria were taken into consideration. The affiliation of the strains tested to species was confirmed with the use of API ID 32 GN tests (bioMerieux), according to bioMerieux procedure.

Evaluation of hydrophobicity of *Ps. aeruginosa* strains. Hydrophobicity of strains was estimated by adsorption to non-polar solvents (hexadecane) – MATH (microbial adhesion to hydrocarbons) according to Doyle & Rosenberg [1990]. In order to perform the test, *Ps. aeruginosa* strains – WZ and MB, were cultured at two temperatures: 4°C and 20°C, on the BHI agar (OXOID). After overnight cultures, the bacteria were washed by centrifugation in 0.85% NaCl. The cell suspension was then standardized to OD_{600} – 0.1 and 0.3 (respectively to incubation temperature) in 0.1 mol/L phosphatic buffer (corresponding to 10^6 and 10^8 cells) using a spectrophotometer. Then, 4.0 mL of the suspension were supplemented with 1.5 mL of hexadecane. All the tests were vortexed for 10 seconds and left intact for 10 min. The procedure was repeated until 60 sec vortexing was reached. Changes in the absorbance of strain suspensions in relation to the non-polar solvent were measured by using a "Carl-zeiss" spectrophotometer at 600 nm. These bacterial strains were incubated at 4°C and 20°C. According to the formula: $A_t/A_o \times 100$ (A_t – preliminary extinction of suspension, A_o – extinction of suspension after defined time of vortexing measured in relation to a blank sample – phosphorus buffer), the results obtained enabled plotting a curve which was a determinant of microorganism affinity to hexadecane [Van der Mei *et al*., 1993]. Strains were considered hydrophilic when the decrease of suspension optical density after a 60-sec vortexing was less than 30%. In the case of a decrease between 30–70% the strains were considered medium hydrophobic. The decrease higher than 70% suggested highly hydrophobic strains.

Evaluation of ability to form biofilm by *Ps. aeruginosa* strains. The ability to form biofilm was tested on Mineral Salt Medium (MSM) base (0.1% KH_2PO_4, 0.1% Na_2HPO_4, 0.05% NH_4NO_3, 0.05% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \times 7H_2O$, 0.002% $CaCl_2 \times 2H_2O$, 0.0002% $FeCl_3$, 0.00002% $MnSO_4$) according to Herman *et al*. [1997]. Bases were inoculated with the tested strains *Ps. aeruginosa* – MB and WZ. The inoculum with the final concentration of 10^2 CFU/mL was added to 100 mL of base. Biofilms were formed on 1.0 cm^2 strerile foils (F1, F3, F5). In 3, 7 and 14-days intervals the ability to form biofilm on different kinds of foils by chosen strains was tested.

To this end particular types of foils were taken from MSM bases and rinsed three times in a physiological saline. Afterwards they were moved into liquid BHI base (Oxoid) with 1% water solution of 2, 3, 5-triphenylotetrazolic chloride. After 24 h the growth of biofilm was checked, which was characterised by pink and reddish tint on the foil surface. Bacteria were rinsed using a 0.5% saponine solution by 15-sec shaking to estimate the number of cells adhered to the surface of the foil [Różalska *et al*., 1998]. After that, quantitative cultures of initial material were prepared on BHI Agar base (Oxoid) and incubated for 72 h at a room temperature. Grown colonies were then counted. The cultures were run in five repetitions.

The influence of *Ps. aeruginosa* strains on gas permeability of foils. Axenic MSM base was inoculated with the tested MB and WZ strains at the final concentration of 10^2 CFU/mL. After that, standardised and axenic foils were submerged in the cultures. The test was performed simultaneously at 4°C and 20°C. After incubation time (7 days), the foils were taken out and rinsed in a 1% solution of sodium azide, dried at a room temperature and taken for further testing. The foils kept in base without microorganisms but treated under the same conditions were used as controls.

The analysis of oxygen permeability (q) through tested foils was performed according to ISO Standard 2556 [ISO, 1974], DIN 53 380 using the OX–TRAN 2/20 ML device (Mocon, USA). The following mixture of gases was used: 98% N_2 and 2% H_2 as carrier gas, and oxygen (purity 3.5) as control gas. Tests were carried out on samples having 50 cm^2 of surface area, at 23°C, under standard conditions of relative humidity 0% and with 100% oxygen concentration. Measurement data were compensated to atmospheric pressure. In order to obtain credible results the samples were conditioned in measuring compartments of the device for no shorter than 5 h.

Statistical analysis. A statistical analysis of the results obtained was carried out using STATISTICA 6.0 PL software. The analysis of statistical significance of differences was performed with the Scheffe test, at a significance level of $p<0.05$. Correlation was set at a significance level of $p<0.005$. An analysis of concentrations of *Ps. aeruginosa* strains depending on their hydrophobicity was performed by the single-link method counted by Euclidean distance (Statistica PL).

RESULTS

Evaluation of hydrophobicity of *Ps. aeruginosa* strains

Statistical record of results distinguished three groups of

different hydrophobicity – hydrophilic, medium hydrophobic and hydrophobic. Statistical evaluation of hydrophobicity variation of *Ps. aeruginosa* MB strains showed a lack of the influence of culture temperature on the tested properties of cell wall. It was demonstrated that in both instances, *i.e.* in incubation at 4°C and at 20°C, the strain tested showed medium hydrophobic properties (Figure 1).

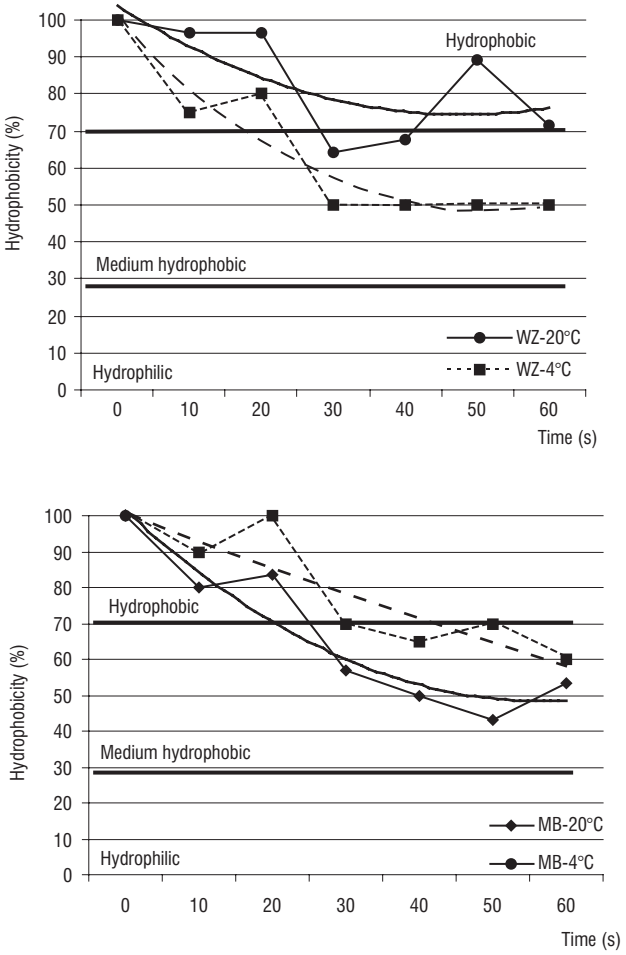


FIGURE 1. Hydrophobicity of *Ps.aeruginosa* strains.

Taking into consideration *Ps. aeruginosa* WZ strain in the culture run at 20° it was found that hydrophilic properties had been kept. The lowering of temperature of the culture to 4°C resulted in the lowering of hydrophilicity of the strain tested (Figure 1).

The ability of adhesion of *Ps. aeruginosa* strains to the surface of the foil

The performed tests, determining the adhesive ability of *Ps. aeruginosa* strains – WZ and MB to chosen food wrapping foils – F1, F3 and F5, showed a lack of significant differences in colonization of the surface by the bacteria (Figures 2, 3). For the tests performed with WZ strain at temperatures of 4°C and 20°C, the assigned variation of function curve did not show any statistically significant differences ($p<0.05$). High resemblance between certain variants of the test was reported (Figure 3). The statistical analysis based on tests with MB strain proved the similarity between the colonization of respective foils and certain temperature (Figure 2).

The intensity of growth of *Ps. aeruginosa* strains – MB and WZ was the highest during the first three days of cultures. In the case of MB strain, the number of defined microorganisms increased from 10^2 to 10^5 CFU/mL, whilst in the case of WZ strain from 10^2 to 10^4 CFU/mL at 20°C and to 10^5 CFU/mL at 4°C (Table 2). During the next days of tests

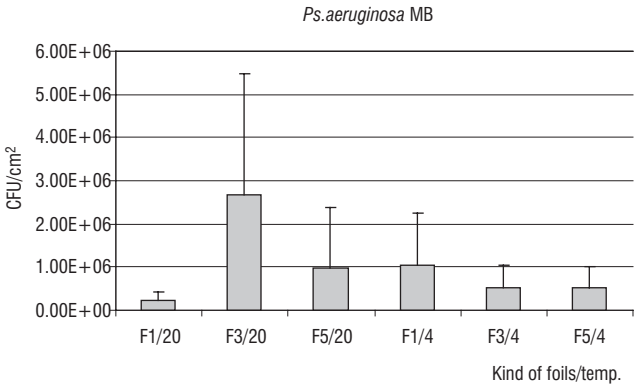


FIGURE 2. The ability to form biofilm by *Ps.aeruginosa* MB.

TABLE 2. Progress in the ability to form biofilm.

Foil labeling	Temp. °(C)	CFU/cm² of *Ps.aeruginosa* WZ			CFU/ cm² of *Ps.aeruginosa* MB		
		3 days	7 days	14 days	3 days	7 days	14 days
F1	20	2.1E+04 (±0.2)	4.7E+04 (± 0.6)	1.7E+05 (±0.12)	2.7E+05 (±0.3)	9.5E+05 (±0.9)	3.7E+06 (±0.6)
F3	20	1.1E+04 (±0.3)	1.6E+04 (±0.4)	2.9E+05 (±0.2)	6.5E+05 (±0.1)	1.4E+06 (± 0.7)	5.2E+07 (± 0.4)
F5	20	1.8E+04 (±0.2)	2.6E+04 (±0.5)	3.1E+05 (±0.34)	4.2E+05 (±0.3)	8.7E+05 (±1.4)	1.9E+07 (± 0.8)
		3 days	7 days	14 days	3 days	7 days	14 days
F1	4	5.0E+05 (±0.5)	1.6E+06 (±1.0)	4.7E+04 (±1.0)	3.2E+05 (±0.4)	1.8E+07 (±0.9)	6.0E+05 (±1.6)
F3	4	1.1E+05 (±0.6)	5.7E+05 (±0.9)	5.8E+06 (±1.0)	5.7E+05 (±0.4)	9.9E+06 (±0.6)	4.0E+05 (±1.6)
F5	4	1.1E+05 (±0.6)	3.9E+06 (±0.8)	2.0E+05 (±1.0)	6.7E+05 (± 0.5)	9.6E+06 (±0.6)	6.3E+05 (±1.1)

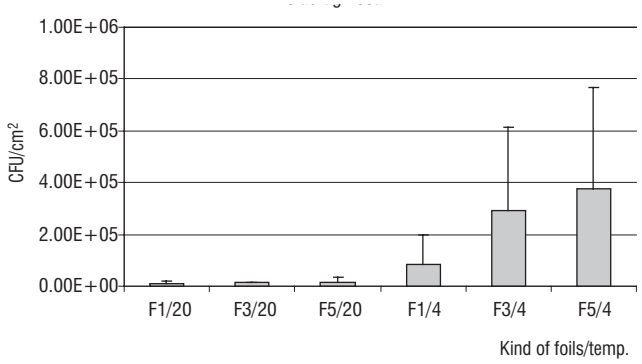


FIGURE 3. The ability to form biofilm by *Ps.aeruginosa* WZ.

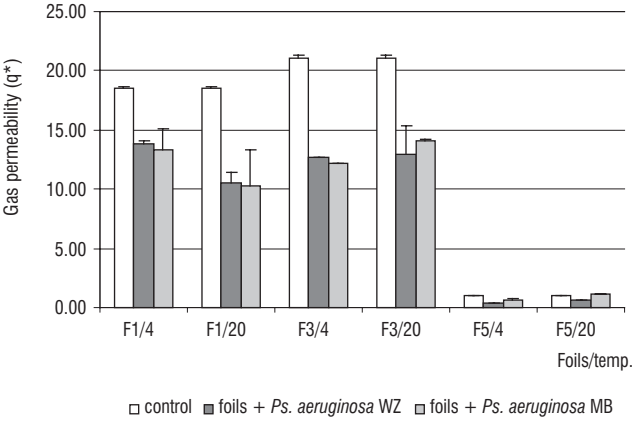
the growth rate of strains on the surface of tested foils was not so intensive (Table 2).

Evaluation of gas permeability through food wrapping foils

Permeability of oxygen through foils submerged only in the base (control sample) and kept under applied testing conditions depended on incubation temperature. It was proved that the usage of cooling temperature changed the properties of foils, which was observed in lower permeability for gases (Figure 4).

As a result of running the culture it was determined that, on the surface of the foils used there occurred a biolayer which in all testing samples additionally reduced oxygen permeability. It was estimated that in cultures with foil F3 at 20°C the average decrease in foil permeability equaled 40% against the control sample. In the case of cultures run at 4°C the results were very close, *i.e.* 38% and 32% for WZ and MB strains, respectively (Figure 4).

In the case of foil F1 such comparable results were not



*q = $cm^3/m^2 \times 24h \times bar$

FIGURE 4. Evaluation of gas permeability through wrapping foils

obtained. The decrease in permeability of gases in all cultures was shown, however it was observed that both strains – WZ and MB at 20° caused an increase in impenetrability by only 14% on average. For foils kept at 4°C the results were higher by 24% (Figure 4).

Slightly different results were obtained for foil F5. In this case, at 20°C the permeability barrier increased by 55% for WZ strain and by 31% for MB strain. At 4°C in the case of WZ strain the permeability was estimated to be 20% lower than that of the control sample. No change was observed for the MB strain (Figure 4).

DISCUSSION

Barrier and mechanical parameters used in food wrapping foils are due to polymers constructing them. Packaged food is usually stored at low temperatures in order to limit the growth of undesirable microflora. Moreover, refrigerating temperatures decrease oxygen permeability through foils [Lisiecki, 2003]. Applied temperatures are not a barrier for metabolic activity of psychrophilous microorganisms. The result of growth of microorganisms is biofilm formation, the enzymatic activity of which can lead to a change of conditions in microbial environment. Taking into consideration *Ps. aeruginosa*, the chemical composition of the surface of this G(-) bacteria directly contributes to colonization of both biological and abiotic surfaces. Particular role is attributed to the three components forming not only the lipopolysaccharide layer (LPS) of the cell but also its hydrophobic activity [Al--Tahan *et al*., 2000]. The base is oligosaccharide layer, which has the unique 2-keto-3-deoxyoctonic acid (KDO), stabilized by Mg^{2+}. An important role in bacterial adhesion is also assigned to antigen O which is bound to irregular endings of oligosaccharide. The process of colonization of abiotic environments by microorganisms, depending on the structure of colonized surfaces, is related with changes in cell wall properties by transformation of proteins and lipids of the outer membrane. The purpose of this process is to increase the affinity to colonized surfaces. Cell wall then becomes more hydrophobic. Bacterial adhesion to rough and matt surfaces is quicker and more intensive. However, the rate of adhesion to smooth and slippery surfaces is much slower at the beginning.

In the food industry the possibilities of forming biofilm are seen as a factor seriously dangerous to the production [Poulsen, 1999; Sharma & Anand 2002b, Magrex-Debar *et al*., 2000]. The most common reported reason for biofilm formation is low hygiene during production and processing process. It concerns mainly meat processing plants, mostly raw packaged products, in which the presence of *Salmonella*, *Campylobacter*, *Yersinia* and *Listeria* is detected [Poulsen, 1999]. Food products packaged in polyethylene foils are also not free from the presence of bacteria responsible for food spoilage. The risks resulting from the presence of undesirable microflora, in the shape of biofilm in production environment, support suggestions to introduce the estimation of biofilm as an important part of HACCP [Sharma & Anand, 2002a]. The control of biofilm formed by microorganisms and its disposal is the greatest problem though [Gilbert *et al*., 2003].

The biofilm formed by *Ps. aeruginosa* is a widely known and commonly described phenomenon [Costerton *et al*., 1999; Xu *et al*., 2001; Daveley & O'Toole, 2000]. The function of such a microbiological structure would not be possible without certain properties of cell wall. The ability to adhere to the surface and its colonization are determined by hydrophobicity of the cell whose activity, together with biofilm incubation, is modified by biosurfactant (surface active

agent) [Al-Tahan *et al*., 2000]. The hydrophobicity tests performed on strains of interest did not show any unequivocal cell preferences. It was decided that at 20°C the reference strain – WZ and the strain isolated from meat – MB belonged to the medium hydrophobic group (Figure 1). The temperature of 4°C, used for the strain isolated from meat, did not have any impact on the change of cell wall properties, whilst it slightly weakened the lipophilic properties of the surface of the cell of *Ps. aeruginosa* – WZ strain (Figure 1). In many works a correlation has been shown between the conditions of culture, including incubation temperature, and hydrophobicity of *Pseudomonas* sp. [Szabo, 2003; Kumar *et al*., 2002; Wolska *et al*., 2002]. The results of our statistical analysis are not convergent with the above reports. The hydrophobicity of the cell is the result of many environmental factors and the application of temperatures yet standard for psychrophilous microorganisms does not necessarily have the influence on the change of its properties. Quantitative assay from cultures developing on the surfaces of tested food wrapping foils suggest the correct interpretation of the results obtained in hydrophobicity tests. Statistically confirmed differences in the rate of foils colonization, in cultures at different temperatures (Table 2), were not observed. The intensive growth of tested microorganisms – WZ and MB strains, on tested food wrapping foils, took place in the first three days of culture (Table 2). This situation has been confirmed by the work of Auerbach *et al*. [2000], which shows that during the first 2–4 days of biofilm growth the cells divide in both upper and lower layer of the biofilm, which is changing with the "age" of biolayer. In further stages the rate of reproduction is lowering (Table 2) due to biofilm structure formation. According to Tuleva *et al*. [2002], the biofilm formation, depending on the strains of *Pseudomonas*, is observed between day 3 and 5 of incubation.

The strains analyzed in this work, *i.e.* WZ and MB, formed biolayer on the surfaces of the foils used (Table 2). *Pseudomonas* sp. belong to the group of psychrotrophic microorganisms responsible for the spoilage of food kept in refrigerating conditions. It is the result of the activity of potent proteolytic and lipolytic enzymes, which leads to deterioration of product quality, and consequently to shortening its shelf life [Jay *et al.,* 2005; Doyle & Rosenberg, 1993]. In modern packaging techniques the wrapping foils should be characterised by very low permeability for oxygen, steam, odorous substances and other gases – CO_2 and N_2. According to Fik & Leszczyńska-Fik [1997], for materials used for vacuum packaging of meat the quotient of oxygen permeability should not exceed 1 mL/m^2/24 h at 23°C. According to Xu *et al*. [2001], the presence and composition of gases, especially available oxygen, used in packaging, is the factor determining the possibility to form bacterial biofilm [Xu *et al*., 1998, 2001].

Food wrapping foils, exposed to bacterial activity, and their evaluation on account of gases permeability, showed that permeability of foils became significantly lower except one sample (Figure 4). Biolayers formed on the surfaces of food wrapping foils caused the occurrence of additional barrier limiting oxygen permeation (Figure 4). Biopolymers which came into existence, as non-toxic biodegradable products, could be used for foil production. However, the main problem would be the elimination of metabolically-active cell from the biofilm formed by them. One of the major function of microbiological biolayer is to protect bacterial structures suspended in matrix against adverse environmental factors [Daveley & O'Toole, 2000]. This is the reason of bacterial resistance in biofilm, for example on antibiotics [Webb *et al*., 2003] or unsuccessful application of some cleaners or purgatives in case of *Pseudomonas* sp. [Poulsen, 1999]. Recent research on the mechanisms of regulation of physiological processes of microorganisms point to the coordination of bacterial behaviour including the activation of defence systems together with biofilm formation [Defoirdt *et al.*, 2004; Magrex-Debar *et al.*, 2000].

CONCLUSIONS

In this work we wanted to determine the effect of the growth of psychrophilous bacteria – *Ps. aeruginosa*, on the structure of food wrapping foils. The results obtained show unequivocally that biofilm formed by the tested microorganisms became the additional barrier for gases. It should be considered to abandon the usage of some synthetic components of foils in favour of natural products.

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WPŁYW TWORZENIA BIOFILMU PRZEZ *PSEUDOMONAS AERUGINOSA* NA PRZEPUSZCZALNOŚĆ GAZÓW PRZEZ FOLIE SPOŻYWCZE

Elżbieta Bogusławska-Wąs[1], Sławomir Lisiecki[2], Anna Drozdowska[1], Katarzyna Ilczuk

[1]Katedra Mikrobiologii Żywności, [2]Zakład Opakowalnictwa i Biopolimerów, Akademia Rolnicza w Szczecinie, Szczecin

Celem niniejszej pracy było określenie wpływu rozwijającego się biofilmu bakteryjnego na przepuszczalność gazów przez folie spożywcze (PA/PE i EVOH) przy wykorzystaniu OX-TRAN 2/20 ML. W doświadczeniu wykorzystano szczepy *Ps. aeruginosa* – wyizolowane z prób mięsa mielonego hermetycznie pakowanego oraz szczepu wzorcowego ATCC. We wszystkich wariantach doświadczenia stwierdzono tworzenie biofilmu przez bakterie. Intensywność kolonizacji powierzchni folii przez testowane szczepy była największa w pierwszych trzech dniach hodowli (tab. 2, 3). Nie stwierdzono korelacji pomiędzy rodzajem folii, a intensywnością tworzonej biofilmu (rys. 2, 3). Ustalono, że powstały biofilm wpływa na podniesienie barierowości wszystkich testowanych folii spożywczych.