Adhesive and hydrophobic properties
of *Pseudomonas aeruginosa* and *Pseudomonas cedrina*
associated with cosmetics

Julia Zabielska*, Alina Kunicka-Styczyńska, Anna Otlewska

Institute of Fermentation Technology and Microbiology, Faculty of Biotechnology and Food Sciences,
Lodz University of Technology, Wolczanska 171/173 St, 90-924 Lodz, Poland,
*e-mail: julia.zabielska@dokt.p.lodz.pl

Abbreviations: TSB, Trypticase Soya Broth; PBS, phosphate buffered saline; TSA, Trypticase Soya Agar; PUM, phosphate urea magnesium sulfate buffer; ATTC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; ISO, International Organization for Standardization; RAPEX, Rapid Alert System.

Abstract. The presence of bacteria in the cosmetic production environment is often connected with non-sterile raw materials, inappropriate production lines disinfection or cross contamination. Among bacteria isolated from the environment, opportunistic pathogens can be also found, posing a risk to patients with lowered immunity. Moreover, their susceptibility to antibiotics and disinfectants is frequently decreased as they develop more complex forms - biofilms. As hydrophobicity and adhesive properties play a vital role in the colonization process the aim of this research was to determine hydrophobic, aggregative and adhesive properties of bacteria isolated from the cosmetics.

Bacteria used in the research were isolated from the body balm and the cosmetic preservative (three strains of *Pseudomonas aeruginosa* and four strains of *Pseudomonas cedrina*) and identified using 16S rRNA gene sequencing. For those strains and also two reference strains (*P. aeruginosa* ATCC15442 and *P. cedrina* DSM17516) an aggregation test, hydrophobicity by two different methods (SAT and MATH) and adhesion to polystyrene by crystal violet binding assay were performed.

According to the SAT method more than half of the tested strains were strongly hydrophobic. Using MATH test, it was proved that four strains (*P. cedrina* DSM17516 and three isolates of *P. aeruginosa*) were strong hydrophobes, however, the rest of the strains expressed moderate hydrophobicity. Moreover, self-aggregation was also observed and for *P. aeruginosa* CFII was more than 20%. All of the strains were able to adhere to polystyrene after 30 minutes contact, almost all of them (excluding *P. cedrina* DSM17516) indicated a moderate adhesion already after four hours of incubation. These results indicate that environmental *Pseudomonas* strains possess strong hydrophobic and adhesive properties, that may results in a colonization of abiotic surfaces.

Key words: *Pseudomonas*, cosmetic contamination, hydrophobicity, adhesion, biofilm.

1. Introduction

Cosmetics may be the source of microorganisms, both pathogenic and non-pathogenic. Apart from the secondary contamination in the course of usage, microbial contamination may take also place during the production processes. Microorganisms may be introduced with raw materials and also from the production line due to its improper disinfection and cross-contaminations. Those irregularities may lead to biodeterioration of products, revealing a change of pH, color, smell, consistency, viscosity, phase separation and gasification (Garbolińska, 2010).

Pseudomonas sp. is a group of Gram-negative bacteria, frequently causing nosocomial infections of patients

with reduced immunity. *P. aeruginosa* belonging to this group is one of the common species associated with infections and are usually very tough to eradicate. Among many virulence factors of *P. aeruginosa* the ones connected with the process of adhesion and biofilm formation (capability of aggregation, cell hydrophobicity, rhamnolipid and EPS production, motility etc.) can be (Briendstien et al., 2011). The ability of colonization of abiotic surfaces by *P. aeruginosa* polymer materials was reported by Zabielska et al. (2015) and the biofilm formed reached more than 6 log CFU/cm² after 24 hours of incubation. Once the biofilm is developed, its removing is much more complicated due to numerous mechanisms such as the presence of matrix or "super resistant" cells – metabolically inactive, grown in the nutrition and oxygen deficiency (Matusiak, 2014).

The purpose of this study was to identify microorganisms isolated from a cosmetic product and a cationic surfactant used as a cosmetic ingredient and determine their hydrophobic, aggregative and adhesive properties, crucial for the biofilm development.

2. Material and methods

2.1. Microorganisms

A total of seven strains were isolated: three *P. aeruginosa* (CFII, CFIVa, CFV) from a body balm and four belonging to *P. cedrina* (DH1, DH2, DN3, DH4) from cetyltrimethyl ammonium chloride, a commercial cationic surfactant used as a cosmetic ingredient. One ml of appropriate product was diluted in 10 ml of saline solution with Tween 80 to neutralize the sample. One ml of each sample was spread on cetrimide agar (BTL, Poland) to allow *Pseudomonas* sp. growth. Strains were initially identified by biochemical tests - API 20E (bioMérieux, France). Additionally, two reference strains were used – *P. aeruginosa* ATCC15442 (American Type Culture Collection) and *P. cedrina* DSM17516 (Deutsche Sammlung von Mikroorganismen). Bacteria were activated in TSB (Merck, Germany) for 24 hours at 30ºC. For phylogenetic analysis the sequences of the following reference strains were used: *P. cedrina* C37, G25, CI-10 originated from milk and clausen (CI-10); *P. aeruginosa* ZM130, KUJM, FQIII originated respectively from textile wastewater, Kalyani sewage treatment plant and sputum.

2.2. Identification of bacterial strains

Bacterial strains were identified based on 16S rRNA gene sequencing. Briefly, genomic DNA was extracted employing the Genomic Mini Kit (A&A Biotechnology, Poland), according to manufacturer's protocol. 16S rRNA gene was amplified with universal primers 27f (5'-AGAGTTT-

GATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTT-GTTACGACTT-3'). PCR reactions were performed using 1.5 U RedTaqReadyMix DNA polymerase (Sigma-Aldrich, USA), 0.4 µL of each primer solution (100µM) and 1 µL of template DNA (50 ng/µL) in the MJ Mini Gradient Thermal Cycler (Bio-Rad, USA). The amplification parameters were used as previously described Kręgiel et al. (2014). The PCR fragments were purified using Clean Up Mini Kit (A&A Biotechnology, Poland). The 16S rRNA sequences were obtained using the BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) and analyzed with the Applied Biosystems model 3730 Genetic Analyzer (Genomed S.A., Poland). The nucleotide sequences of 16S rRNA were proofread, assembled and compared with sequences available in The National Center for Biotechnology Information (NCBI) using basic local alignment search tool BLAST 2.6.0 (Zhang et al., 2010). The nucleotide sequences of 16S rRNA gene were deposited in GenBank of the NCBI under the following accession numbers: KY045833-KY045836 (*P. cedrina*) and KY045837-KY045839 (*P. aeruginosa*).

2.3. Phylogenetic analysis

The phylogenetic tree was constructed by neighbor-joining method using the MEGA7 software (Saitou & Nei, 1987; Kumar et al., 2016). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura et al., 2004). The analysis involved 13 nucleotide sequences of 16S rRNA genes from the NCBI database. All positions containing gaps and missing data were eliminated. There was a total of 1384 positions in the final dataset.

2.4. Aggregation test

Aggregation of strains was evaluated by the method proposed by Del Re et al. (2000). Bacteria were grown in TSB (Merck, Germany) for 24 hours at 30ºC. Two ml of the culture was centrifuged for 10 minutes (5000 rpm). The precipitate was suspended in two ml of 10 µM PBS buffer and the absorbance at the wavelength 600 nm was measured (A). Subsequently, the suspension was incubated for two hours at 30ºC and the absorbance of the upper layer was measured (A_0). Aggregation was calculated according to the formula:

$$\text{Aggregation (\%)} = \frac{1 - A}{A_0} \times 100$$

2.5. Hydrophobicity – MATH and SAT assay

Hydrophobic properties of the cell surface were determined by two different methods SAT (Salt Aggregation Test) and MATH (Microbial Adhesion to Hydrocarbon).

In the SAT method, activated bacteria were inoculated on TSA (Merck, Germany) and incubated for 24 hours at 30ºC. After that, bacteria inoculum in 2 μM phosphate buffer saline (PBS, Merck, Germany) was prepared (approx. 2º in McFarland scale) and mixed with ammonium sulfate of different molarity (from 0.1 to 4) in a ratio 1:1. The control included bacteria inoculum in PBS buffer. After 15–20 minutes, the aggregates formation was evaluated by macroscopic observations and classified according to the scale proposed by Nwanyanwu and Abu (2013), in which strains were divided into three categories: of high hydrophobicity (<1.0 M), of moderate hydrophobicity (1.0–2.0 M) and of low hydrophobicity (>2.0 M).

In the MATH assay with some modifications (Rosenberg, 1984), bacteria were activated on TSA slants (incubation 24 hours, 30ºC). Then, inoculum (A_1) was prepared either in 10 μM phosphate buffer saline (PBS), or in phosphate urea magnesium sulfate buffer (PUM, Merck, Germany). Two and half ml of inoculum was mixed with half ml of *p*-xylene and incubated for 10 minutes at 37ºC. Thereafter, samples were homogenized and incubated once again for 45 minutes. The samples were allowed to be separated into two phases (aqueous and hydrocarbon). The aqueous phase (A_2) was measured spectrophotometrically at the wavelength 520 nm. The hydrophobicity index (percentage of cell adhesion to hydrocarbon) was calculated upon the following formula:

$$A (\%) = \frac{A_1 - A_2}{A_1} \times 100$$

The results were evaluated according to the scale (Kadam et al., 2009): strongly hydrophobic (>50%), moderate hydrophobic (20–50%) and low hydrophobic (<20%).

2.6. Adhesion to polystyrene

Adhesion to polystyrene was carried out by the method proposed by Stepanovic et al. (2000) with some modifications. Bacteria were cultivated in TSB (Merck, Germany) for 24 hours at 30ºC. 20 μl of bacteria was carried into 230 μl of TSB on 96-well microtitration plate. Plates were incubated in different periods of time – for 0.5, 4 and 24 hours, then the content was poured out and wells were rinsed with sterile water. The plates were dried and adhered bacteria cells were fixed with 96% ethanol for 20–30 minutes. Afterwards, ethanol was removed and bacteria were stained with 0.5% crystal violet. The dye was removed and the plates were again rinsed with water. After drying, each well was decolorized with 96% ethanol and the absorbance was measured (λ=570 nm). The blank sample was the growth medium.

2.7. Statistical analysis

The tests were conducted in triplicate and SD (standard deviation) was calculated using ORIGIN LAB 6.1 version (Northampton, USA). The correlation between buffers used in MATH method was evaluated by Pearson's coefficient analysis.

3. Results and discussion

Seven strains belonging to *Pseudomonas* species were isolated from the body balm and the cosmetic preservative. Genetic identification based on 16S rRNA gene sequencing showed that three strains (CFII, CFIVa and CFV) are classified as *P. aeruginosa* and four (DH1, DH2, DH3 and DH4) as *P. cedrina.* Phylogenetic analysis, presented in the Figure 1, showed a 100% similarity to the type strains.

The presence of unfavorable microflorawas described also by Campana et al. (2006). They investigated 91 commercial cosmetics and opportunistic strains such as *Pseudomonas putida* and *Staphylococcus aureus* were found in some samples. According to the different source - RAPEX data for non-food products in the EU, only in the last decade over 100 cases of microbiological contaminations were noted, including the presence of opportunistic pathogens such as *P. aeruginosa*, *Burkholderia cepacia* and *S. aureus*. Also Birketsoz et al. (2013) indicated the contamination of *P. aeruginosa, P. putida* and *Pseudomonas fluorescens* in commercial, unopened cosmetic products.

In the course of the study aggregative, adhesive and hydrophobic properties of the identified *Pseudomonas* sp. strains were assessed. Aggregation and hydrophobic abilities expressed by *Pseudomonas* strains were presented in Table 1. and Figure 2. The highest values of aggregation was presented by environmental *P. aeruginosa* strain CFII and it reached 20.74%. The aggregation of the majority of strains fluctuated from 9 to 13%. The least aggregative strain was the reference strain *P. aeruginosa* ATCC15442 (4.82%). Aggregation of different pathogens such as *Salmonella typhimurium*, *Listeria monocytogenes, Staphylococcus aureus* and *Shigella boydii* was described by Xu et al. (2010). The values obtained by Xu et al. (l.c.) were similar and oscillated from 5 to 23% for bacteria in PBS buffer, however after addition of NaCl in different concentration aggregation were much higher. The aggregation is connected with cell-to-cell binding and as it was showed, environmental conditions have a great influence on it.
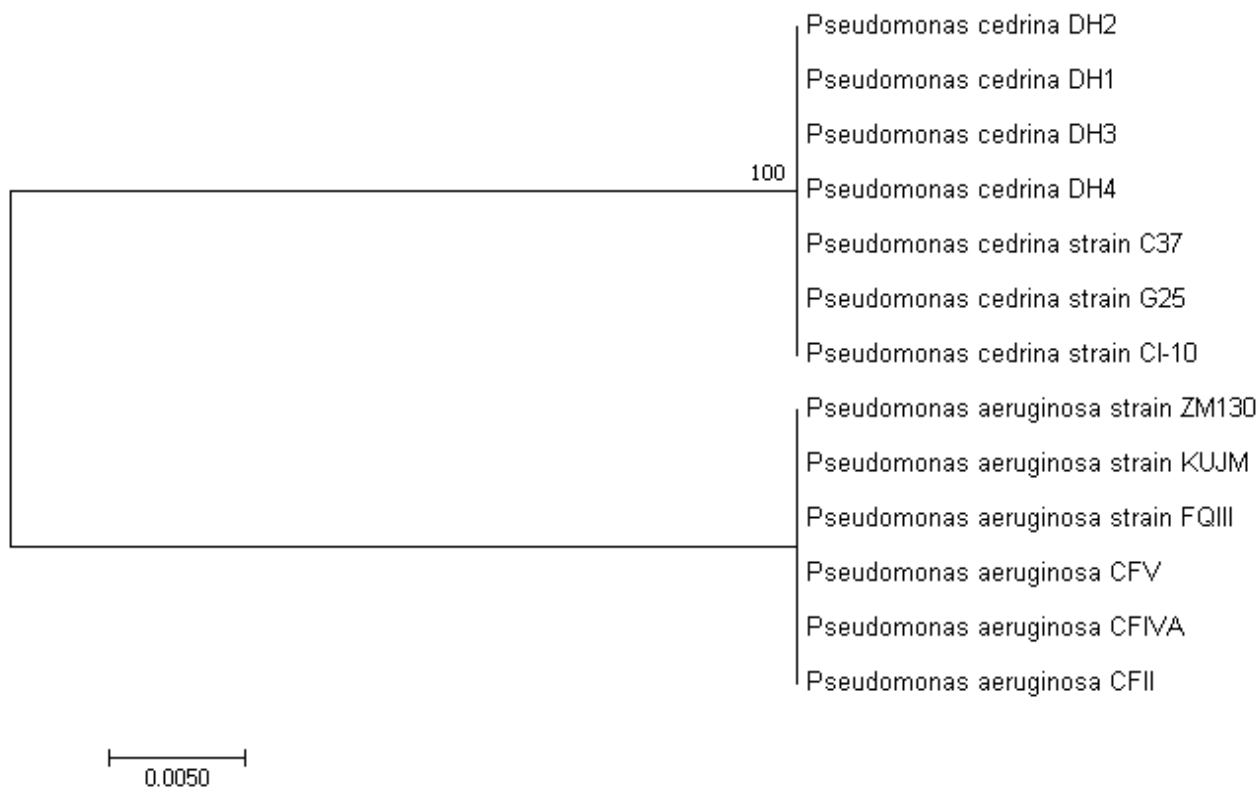
```
100
```

```
0.0050
```

Figure 1. Phylogenetic tree of *Pseudomonas* sp. cosmetic contaminant: *P. aeruginosa* CFII, CFIVa, CFV isolated from a body balm; *P. cedrina* DH1, DH2, DN3, DH4 isolated from cetyltrimethyl ammonium chloride; *P. cedrina* C37, G25, CI-10 originated from milk and clausen (CI-10); *P. aeruginosa* ZM130, KUJM, FQIII originated respectively from textile wastewater, Kalyani sewage treatment plant and sputum

Hydrophobicity of isolated bacteria was determined by two methods. The first one – Salt Aggregation Test – indicated that all of the strains were high or moderate hydrophobes. In comparison to environmental isolates, reference strains were less hydrophobic (Table 1). The same method was used by Wolska et al. (2002) for clinical strains of *P. aeruginosa*. More than half of the tested bacteria expressed strong hydrophobic properties. Result of *Pseudomonas* sp. hydrophobicity using MATH method were presented in Figure 2. *P.* cedrina was characterized by the highest hydrophobicity index, which reached respectively 88.74% in PBS buffer and 89.62% in PUM buffer, and was classified as strongly hydrophobic. *P. aeruginosa* isolates (CF) expressed also a strong hydrophobicity with index values oscillated between 60–80%. The lowest index values were obtained for *P. cedrina* strains (DH 1–4) and for the reference strain *P. aeruginosa* ATCC15442, what classified them as moderate hydrophobes.

Moreover, the degree of hydrophobicity evaluated by Pearson's coefficient analysis showed strong correlation

Table 1. *Pseudomonas* sp. strains aggregation and degree of cell surface hydrophobicity assessed by *SAT method

Microorganism		Aggregation (%)	Hydropho-bicity*
Pseudomonas cedrina	DSM17516	11.57±3.64	moderate
	DH1	11.80±3.43	high
	DH2	10.50±4.11	moderate
	DH3	9.43±0.86	high
	DH4	10.58±1.47	high
Pseudomonas aeruginosa	ATCC15442	4.82±0.11	moderate
	CFII	20.74±1.66	high
	CFIVa	12.94±0.47	high
	CFV	11.00±1.87	moderate

between both buffers tested. Norouzi et al. (2010) also presented data regarding *P. aeruginosa* strains hydrophobicity assessed by the MATH method and the majority of strains demonstrated moderate hydrophobic properties. Research conducted by Kadam et al. (2009) on environmental strains *P. aeruginosa* and *P. fluorescens* indicated that both strains are moderate hydrophobic in PBS buffer (28.8% and 36.4% respectively) and low hydrophobic in PUM buffer (18% and 15.2% respectively). Tyfa et al. (2015) tested Gram-positive *Alicyclobacillus* sp. using the same method and almost all of them were strong hydrophobes in PUM buffer and majority of strains were moderate hydrophobes in PBS buffer.

The crystal violet binding method was used to assess *Pseudomonas* sp. strains capability of adhering to polystyrene and creating biofilm, thus to evaluate their adhesive properties (Fig. 3). All of the strains were able to adhere to the polystyrene surface after 0.5 hour contact and the highest absorbance values were reached for *P. cedrina* isolates (DH1–4). After 4 hours of contact for almost all strains absorbance ranged from 0.12 to 0.22 (excluding *P. cedrina* DSM17516), which indicated on their moderate adherence. 24-hour incubation showed that strains *P. cedrina*: DH3, DH1 and *P. aeruginosa* ATCC15442 expressed a great adhesion. For *P. cedrina* DSM17516 reference strain the ab-
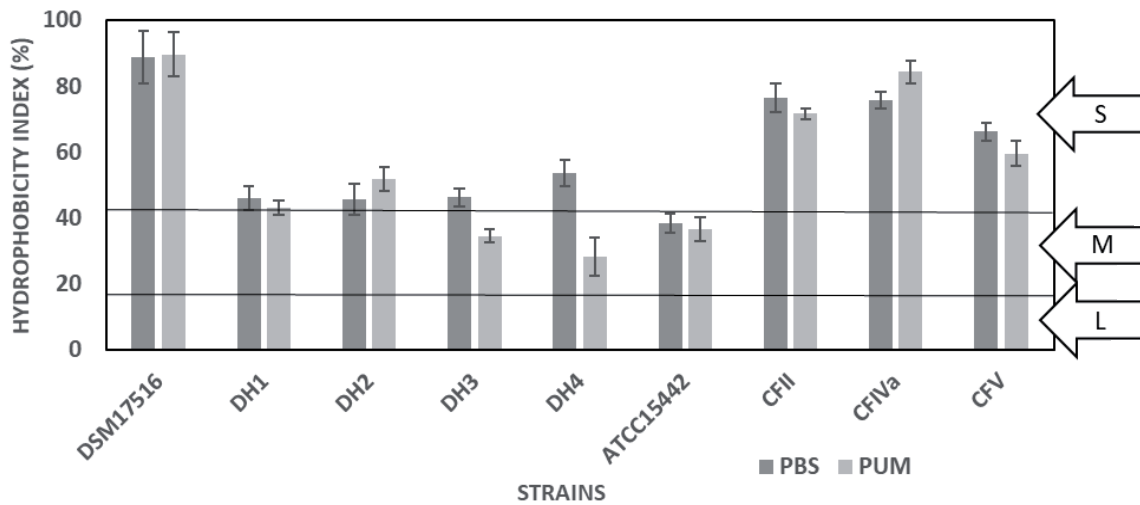

Figure 2. *Pseudomonas* sp. cosmetic contaminants hydrophobicity index estimated by the MATH test in PBS and PUMbuffers, classified as: S – strongly hydrophobic, M – moderate hydrophobic and L – low hydrophobic
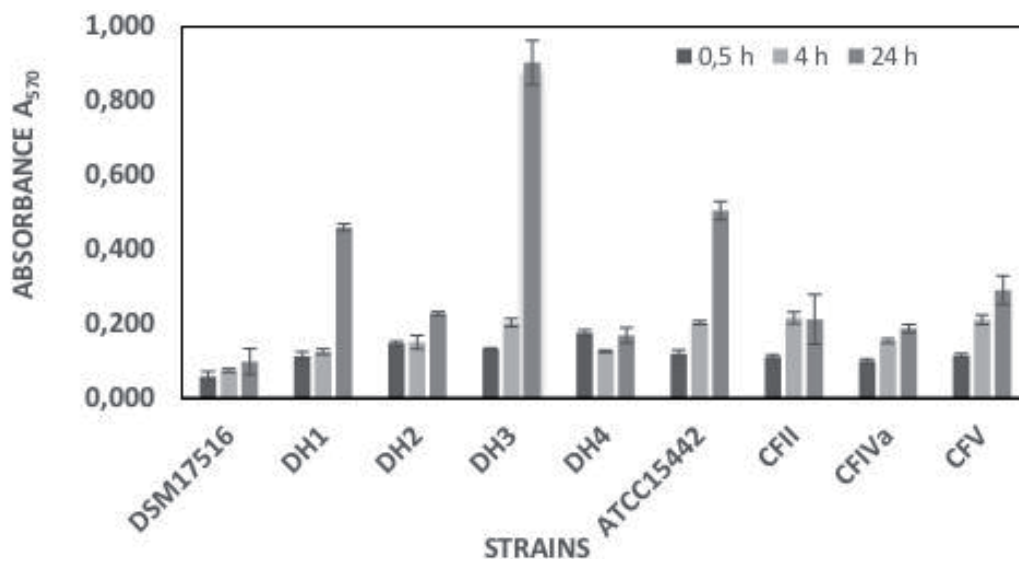

Figure 3. *Pseudomonas* sp. cosmetic contaminants adhesion to polystyrene within 0.5–24 hours of incubation

sorbance reached 0.097 which indicated on weak adhesion. The rest of strains expressed moderate adhesion. Similar results to *P. cedrina* DMS17516 was reported by Sedláčková et al. (2011) for *Pseudomonas fluorescens* reaching after 0.5 hour incubation the absorbance equal 0.087.

All in all, cosmetics can be the source of opportunistic pathogens which pose a health threat to humans with impaired immune system. In pursuance of the ISO standard (PN-EN ISO 17516: 2014-11), the presence of microbes such as *P. aeruginosa, S. aureus* and *Candida albicans* is not allowed in one gram of the cosmetic product, however these microorganisms are often found in cosmetics or their ingredients, which was proved in this report. What is more, such bacteria often present a great hydrophobic and adhesive properties which can lead to colonization of abiotic surfaces and biofilms creation.

References

Birteksoz A.R., Tuysuz M. & Otuk G., 2013, Investigation of preservative efficacy and microbiological content of some cosmetics found on the market. Pakistan Journal of Pharmaceutical Sciences 26: 153–157.

Briedenstein E.B.M., De la Fuente-Nunez C. & Hancock R.E.W., 2011, *Pseudomonas aeruginosa*: all roads lead to resistance. Trends in Microbiology 19: 419–426.

Campana R., Scesa C., Patrone V., Vittoria E. & Baffone W., 2006, Microbiological study of cosmetics product during their use by consumers: health risk and efficacy of preservative systems. Letters in Applied Microbiology 43: 301–306.

Del Re B., Sgorbati B., Miglioli M. & Palenzona D., 2000, Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Letters in Applied Microbiology 31: 438–442.

Garbolińska M., 2010, Microbiological control in the cosmetic industry. Świat Przemysłu Kosmetycznego 2: 26–29.

Kadam T.A., Rupa L., Balhal D.K., Totewad N.D. & Gyananath G., 2009, Determination of the degree of hydrophobicity – A technique to assess bacterial colonization on leaf surface and root region of lotus plant. Asian Journal of Experimental Sciences 23(1): 135–139.

Kręgiel D., Otlewska A. & Antolak H., 2014, Attachment of *Asaia bogorensis* originating in fruit-flavored water to packaging materials. BioMed Research International. (http://dx.doi.org/10.1155/2014/514190).

Kumar S., Stecher G. & Tamura K., 2016, MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33: 1870–1874.

Matusiak D.M., 2014, Zakażenia układu moczowego z udziałem *Proteus mirabilis* – rola biofilmu i inkrustacji cewnika urologicznego [Urinary tract infections caused by *Proteus mirabilis* – role of the biofilm and the encrustation of the urological catheter]. Postępy Mikrobiologii 53(2): 173–181.

Nwanyanwu C. & Abu G., 2013, Influence of growth media on hydrophobicity of phenol-utilizing bacteria found in petroleum refinery effluent. International Research Journal of Biological Sciences 2: 6–10.

Norouzi F., Mansouri S., Moradi M. & Razavi M., 2010, Comparison of cell surface hydrophobicity and biofilm formation among ESBL- and non-ESBL-producing *Pseudomonas aeruginosa* clinical isolates. African Journal of Microbiology Research 4(11): 1143–1147.

PN-EN ISO 17516: 2014-11 Cosmetics-Microbiology-Microbiological limits.

Rosenberg M., 1984, Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiology Letters 22: 289–295.

Saitou N. & Nei M., 1987, The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406–425.

Sedláčková P., Čeřovský M., Horsáková I. & Voldřich M., 2011, Cell surface characteristic of *Asaia bogorensis* – spoilage microorganism of bottled water. Czech Journal of Food Sciences 29(4): 457–461.

Stepanovic S., Vukovic D., Dakic I., Savic B. & Svabic-Vlahovic M., 2000, A modified microtiter-plate test for quantification of staphylococcal biofilm formation. Journal of Microbiological Methods 40(2): 175–179.

Tamura K., Nei M. & Kumar S., 2004, Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 101:11030–11035.

Tyfa A., Kunicka-Styczyńska A. & Zabielska J., 2015, Evaluation of hydrophobicity of quantitative analysis of biofilm formation by *Alicyclobacillus* sp. Acta Biochimica Polonica 62(4): 785–790.

Xu H., Zou Y., Lee H.-Y. & Ahn J., 2010, Effect of NaCl on the biofilm formation by foodborne pathogens. Journal of Food Science 75(9): 581–585.

Wolska K., Pogorzelska S., Fijoł E., Jakubczak A. & Bukowski K., 2002, Influence on growth conditions on cell surface hydrophobicity of *Pseudomonas aeruginosa*, Medycyna Doświadczalna i Mikrobiologia 54: 61–66.

Zabielska J., Kunicka-Styczyńska A., Rajkowska K. & Tyfa A., 2015, Opportunistic Gram-negative rods' capability of creating biofilm structures on polivynyl chloride and styrene-acronitrile copolymer surfaces. Acta Biochimica Polonica 62(4): 733–737.

Zhang Z., Schwartz S., Wagner L. & Miller W., 2000, A greedy algorithm for aligning DNA sequences. Journal of Computational Biology 7: 203–214.