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Regular paper

Evaluation of hydrophobicity and quantitative analysis of biofilm formation by *Alicyclobacillus* sp.

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***Alicyclobacillus* sp. are acidothermophilic bacteria frequently contaminating fruit based products (juices and juice concentrates). These sporulating bacteria are able to survive at elevated temperatures and highly acidic environments which causes difficulties in their removal from industrial environments. Although numerous literature data examine *Alicyclobacillus* sp. presence in fruit based products and methods of their elimination, there is still a limited knowledge on ability of these bacteria to adhere to abiotic surfaces. Therefore, the objective of this study was to determine *Alicyclobacillus* sp. cells' hydrophobicity and capability of biofilm formation on a glass surface. The degree of cells hydrophobicity, according to Microbial Adhesion to Hydrocarbon (MATH) and Salt Aggregation Test (SAT), was investigated for eleven environmental isolates from natural Polish habitats, identified as *Alicyclobacillus* sp., and a *Alicyclobacillus acidoterrestris* DSM 3922 reference strain. The dynamics of biofilm formation within 3-day incubation on a glass surface was evaluated and quantified by a plate count method both, for cultures with and without agitation. All of the bacterial strains tested expressed ability to colonize a glass surface and four environmental isolates were classified as fast-adherent strains. The mature biofilm structures were predominantly formed after 48 hours of incubation. Dynamic culturing conditions were observed to accelerate the biofilm formation. The majority of strains expressed a moderate hydrophobicity level both, in SAT (41.7%) and MATH-PBS (75.0%), as well as MATH-PUM (91.7%) tests. However, no correlation between hydrophobicity and cell adherence to a glass slide surface was observed.**

Key words: *Alicyclobacillus* sp., acidothermophilic bacteria, biofilm, hydrophobicity, glass surface

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INTRODUCTION

The phenomenon of biofilm formation is a multi-step process dependent on environmental conditions, contact surface properties and extracellular polymers secreted by bacteria (Kolwzan *et al*., 2011; Myszka & Czaczyk, 2011; Srey *et al*., 2013). The distance between bacterial cells and a solid surface, as well as electrostatic charge and surface hydrophobicity, play a pivotal role in bacterial colonization of the material (Rosenberg *et al*., 1981; Myszka & Czaczyk, 2011; Olszewska *et al*., 2013). The initial stage involves planktonic cell attachment, however, the strength of cell-to-surface binding is weak enough to cause reversibility of the process. Cell adhesion, followed by the production of extracellular polymers (EPS), is regarded as an irreversible process and early formation of biofilm. Subsequent development of microcolonies enmeshed within an EPS matrix, leads to gradual bacterial multiplication and diversification of cell functions (Kolwzan *et al*., 2011; Myszka & Czaczyk, 2011; Olszewska *et al*., 2013). Moreover, the mature biofilm acquires higher resistance towards external factors and antimicrobial agents (Stewart *et al*., 2002; Shi *et al.,* 2009). Continuous increase of biofilm layers induces detachment and dispersal of biofilm-associated cells and their adhesion onto new locations.

Bacterial biofilms remain a serious problem especially for food industry (Garret *et al.,* 2008; Olszewska *et al*., 2013; Srey *et al*., 2013). Colonization of industrial installation surfaces may lead to potential cross-contamination of products, decrease in their shelf-life stability and inconsequence food product spoilage. Biofilm formation by pathogenic microorganisms should be monitored in particular, as their contact with food-surfaces increase the risk of diseases transmission (Kolwzan *et al*., 2011; Olszewska *et al*., 2013).

Since 1982, fruit processing industry has been struggling with the presence of acidophilic thermophilic bacteria, even in aseptically packed fruit juices and concentrates (Cerny *et al*., 1984; Splittstoesser *et al*., 1994; Tianli *et al*., 2014). Such a contamination is associated with a specific type of bacteria belonging to the *Alicyclobacillus* genus. Alicyclobacilli are regarded as Gram-positive, aerobic and sporulating bacteria, able to survive in low pH and at elevated temperatures (Smit *et al.,* 2011). The major components of their cell membranes are cyclic fatty acids which are responsible for a relatively high resistance towards inconvenient conditions (Hippchen *et al*., 1981; Wisotzkey *et al*., 1992; Walls & Chuyate, 1998). As soil-borne microorganisms, *Alicyclobacillus* sp. might contaminate surface of fruits and thus be transferred into industrial processing plants. Spores are resistant enough to withstand commercial pasteurization which activates spore germination in the final product (Chang & Kang, 2004; Sokolowska *et al*., 2014). Contaminated juices and concentrates manifest by a specific medicinal or phenol-like scent due to metabolites produced, like guaiacol and halophenols (Smit *et al.,* 2011). Unfortunately, although current knowledge considers the presence of

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Abbreviations: MATH, Microbial Adhesion to Hydrocarbon; SAT, Salt Aggregation Test; PBS, phosphate buffered saline; PUM, phosphate urea magnesium sulfate buffer; BAT medium, *Bacillus acidoterrestris* medium

Alicyclobacillus sp. cells and spores in fruit juices and methods of their elimination, there is still limited data on their abiotic surface colonization ability. Only few studies considering *A. acidoterrestris* adhesion to abiotic surfaces have been undertaken, and then only the reference strains were mainly being investigated (Podolak *et al.*, 2009; dos Anjos *et al.*, 2013; Shemesh *et al.*, 2014). Because of the high diversity of alicyclobacilli originated from different regions of the world, it would be justified to characterize the biofilm formation ability of the native *Alicyclobacillus* isolates (Groenewald *et al.*, 2008; Tianli *et al.*, 2014). Although Polish fruit and vegetable processing industry struggles with products contamination by *Alicyclobacillus* sp., studies considering environmental isolates of these bacteria have not been considered so far. Therefore, in this study the cell hydrophobicity and the dynamics of biofilm formation on a glass surface of the environmental *Alicyclobacillus* sp. strains, isolated from natural Polish habitats, were determined. Following the trends for organic foods and environment protection, the consumers' preferences are diverted to recycling glass juice packaging. According to AIJN European Fruit Juice Association (2014) glass containers account for 25.3% of total fruit juice and nectars' packaging on Polish market, nearly twice more than plastic bottles. In the light of juice quality assurance, the glass surface colonization by alicyclobacilli is a great concern of producers.

MATERIALS AND METHODS

Microorganisms and culture conditions. Eleven environmental isolates previously characterized as *Alicyclobacillus* spp. and an *Alicyclobacillus acidoterrestris* DSM 3922 reference strain, provided by the German Collection of Microorganisms and Cell Culture (Deutsche Sammlung von Mikroorganismen und Zellkulturen), were used. Environmental strains were isolated from garden soil (042, 057), bark of apple trees (009, 041, 056), and surface of apples (007, 008, 024, 025, 040, 055), collected from different areas located near the city of Lodz (Poland). Bacterial strains were activated in *Bacillus acidoterrestris* broth and *Bacillus acidoterrestris* agar media (BAT) (Merck, Darmstadt, Germany) for 24 hours at the temperature of 44°C. Vegetative cells from early log phase, prior to spore formation, were used for the experiment and standardized in BAT broth (0.5 g/l $CaCl_2$, 1.0 g/l $MgSO_4 \times 7H_2O$, 0.4 g/l $(NH_4)_2SO_4$, 6.0 g/l KH_2PO_4, 4.0 g/l yeast extract, 5.0 g/l glucose, 2.0 ml trace elements solution (0.66 g/l $CaCl_2 \times 2H_2O$, 0.18 g/l $ZnSO_4 \times 7H_2O$, 0.16 g/l $CuSO_4 \times 5H_2O$, 0.15 g/l $MnSO_4 \times H_2O$, 0.18 g/l $CoCl_2 \times 5H_2O$, 0.1 g/l H_3BO_3, 0.3 g/l $Na_2MoO_4 \times 2H_2O$; pH 4.0)) to final inoculum of approximately 3.5-4.0 log units per milliliter.

Bacterial hydrophobicity determination. Cell surface hydrophobicity was assessed by two protocols for modified Microbial Adhesion to Hydrocarbon (MATH) and Salt Aggregation Test (SAT). MATH Assay was carried out according to the procedure described elsewhere with some modifications (Rosenberg *et al.*, 1984; Nwanyanwu & Abu, 2013). Bacterial cells were suspended (A_1) in sterile capped test tubes containing 2.5 ml phosphate buffered saline (PBS, 0.02 M; pH 7.2) or 2.5 ml phosphate urea magnesium sulfate buffer (PUM, 22.2 g/l $K_2HPO_4 \times 3\ H_2O$, 7.26 g/l KH_2PO_4, 1.8 g/l urea, 0.02 g/l $MgSO_4 \times 7H_2O$; pH 7.1). Thereafter, 0.5 ml of *p*-xylene was added and the mixtures were incubated at 44°C for 10 minutes. Samples were homogenized for 60 s and further incubated for 1 hour. Afterwards, the hydrocarbon and aqueous phases of the mixtures were allowed to separate and the optical density of aqueous phase was measured spectrophotometrically at 600 nm (A_2). The percentage of cell adhesion to hydrocarbons was calculated upon the following formula: $A\ (\%) = [(A_1 - A_2)/A_1] \times 100\%$. The degree of a strain's hydrophobicity was assigned as strongly hydrophobic, moderately hydrophobic and hydrophilic within percentage adhesion values equal $>50\%$, 20–50% and $<20\%$ respectively (Kadam *et al.*, 2009). Salt Aggregation Test was carried out as described by Nwanyanwu & Abu (2013) with some modifications. The principle of the SAT assay is based on cell precipitation in the presence of salts (Ljungh & Wadström, 1982). Bacterial culture suspensions in phosphate buffered saline (PBS, pH 6.8) and ammonium sulfate solutions $(NH_4)_2SO_4$ of molarities ranging from 0.1 to 4.0 were prepared. Then, 50 µl of cell suspension was mixed with an equal volume of the salt solution. The lowest $(NH_4)_2SO_4$ molarity giving visual bacterial clumping after 4 minutes was considered as the SAT value. Classification of hydrophobicity was expressed as strongly hydrophobic, hydrophobic and hydrophilic for <1.0 M, 1.0–2.0 M, >2.0 M, respectively (Nwanyanwu & Abu, 2013). Cell precipitation without the presence of ammonium salt was assigned as autoaggregation.

Cell adherence and biofilm quantification. Adhesion of *Alicyclobacillus* sp. cells was carried out similarly to the technique described elsewhere with modifications (Ortega *et al.*, 2008; dos Anjos *et al.*, 2013). Prior to sterilization, each bottle containing 45.5 ml of BAT broth was equipped with a glass slide previously washed and sanitized twice using a protocol by Marques *et al.* (2007). Each microbial strain being examined was inoculated and incubated for 4, 24, 48 and 72 hours at 44°C both, in cultures with (60 rpm) and without agitation. For each sample, the biofilm was collected by swabbing; loosely attached cells (non-adherent cells) were rinsed with a saline solution and the remaining adhered cells (sessile cells) were collected by swabbing. The biofilm, non-adherent cells and sessile cells were estimated by a plate count method on BAT agar medium (44°C, 24 hours). Results are presented as log CFU/cm^2.

Statistical analysis. Each sample was tested in triplicate and the average and standard deviation were calculated by means of STATISTICA version 6.0 Pl. The correlation between cell surface hydrophobicity for applied buffers was evaluated by Pearson's coefficient analysis.

RESULTS AND DISCUSSION

Results of *Alicyclobacillus* sp. cell hydrophobicity assessment using MATH and SAT methods are presented in Tables 1 and 2. The degree of cell hydrophobicity in MATH assay varies among buffers and bacterial strains used. Majority of the isolates and *A. acidoterrestris* DSM 3922 reference strain did not exhibit any statistically important differences within applied buffers. Environmental isolates 009 and 041 showed weaker hydrophobicity in phosphate buffered saline (PBS) when compared to phosphate urea magnesium sulfate buffer (PUM) (Table 1). On the contrary, isolate 024 was observed to increase its hydrophobicity level in the PBS buffer. Isolate 008 appeared to be a moderately hydrophobic strain in the presence of phosphate buffered saline and rather a hydrophilic strain in PUM buffer instead. Considering results obtained from the SAT assay, three isolates (007, 025 and 042) expressed an autoaggregation tendency

Table 1. *Alicyclobacillus* sp. cell surface hydrophobicity assessed by MATH and SAT assays.

Strain	MATH [%]		SAT
	PBS	PUM	
DSM 3922	47.5±2.2	44.2±4.6	1.5
007	31.5±6.7	46.1±4.5	autoaggregation
008	37.1±0.3	11.9±0.8	0.1
009	15.9±0.7	34.3±2.5	1.5
024	50.6±0.6	38.4±1.0	1.0
025	42.6±0.8	32.2±0.3	autoaggregation
040	25.5±0.5	22.6±3.9	3.0
041	17.3±1.8	33.3±4.7	1.0
042	41.3±1.8	26.9±5.2	autoaggregation
055	40.0±0.1	34.3±3.3	2.0
056	48.9±0.8	38.3±0.1	0.1
057	47.7±1.0	42.7±1.3	0.1

MATH — Microbial Adhesion to Hydrocarbons; SAT — Salt Aggregation Test; PBS — phosphate buffered saline; PUM — phosphate urea magnesium sulfate buffer

Table 2. Degree of hydrophobicity of *Alicyclobacillus* sp. estimated by different tests

Assay	Criteria	Hydrophobicity	Number of bacterial strains (%)
MATH (PBS)	>50%	strongly hydrophobic	8
	20–50%	moderately hydrophobic	75
	<20%	hydrophilic	17
MATH (PUM)	>50%	strongly hydrophobic	0
	20–50%	moderately hydrophobic	92
	<20%	hydrophilic	8
SAT	0M	autoaggregation	25
	<1.0M	strongly hydrophobic	25
	1.0–2.0M	hydrophobic	42
	>2.0M	hydrophilic	8

(Table 1). Moreover, only isolate 040 appeared to be hydrophilic, while isolates 008, 056 and 057 were strongly hydrophobic. All of the remaining strains expressed moderate hydrophobicity. Although all of the twelve *Alicyclobacillus* sp. tested strains expressed variations in the degree of hydrophobicity, majority of them showed a moderate hydrophobicity both, for SAT (41.7%) and MATH-PBS (75.0%), as well as MATH-PUM (92.7%) assays (Table 2).

Variations in the cells' hydrophobic character using MATH and SAT might stem from different criteria evaluated by both methods. The SAT values are believed to depend on microbial culture's age, as well as the diversity of cells' outer membrane composition and cell charge (Kadam *et al*., 2009; Nwanyanwu & Abu, 2013). In contrast, buffers and hydrocarbons used for MATH assay seem to play a particular role for determination of hydrophobicity. Not only the *p*-xylene is reported to exhibit toxic and destructive effect on microbial cells, but also the ionic strength of PUM buffer is associated with greater hydrophilicity. In the present study, the different effect on cells' hydrophobic character assessment between the applied buffers was confirmed. Moreover, the degree of hydrophobicity for selected *Alicyclobacillus* sp. environmental isolates and *A. acidoterrestris* DSM 3922 evaluated by Pearson's coefficient did not show any consistent correlation. Similar results, however, obtained for other microorganisms have been reported by other authors (Basson *et al*., 2007; Kadam *et al*., 2009; Qiao *et al*., 2012; Nwanyanwu & Abu, 2013).

The capability of *Alicyclobacillus* sp. adherence to glass surface and formation of biofilm within 3-day incubation at 44°C was investigated. The extent of biofilm formed varied among the strains (Fig. 1). Apart from three isolates (025, 040, 056), the amount of biofilm formed by almost all of the tested alicyclobacilli was greater (about 20.1–34.1%) when grown in agitated cultures in comparison to the non-agitated culture. Our results are in disagreement with observations made by Basson *et al.* (2007) for *Flavobacterium johnsoniae* environmental isolates.

Research on biofilm formation dynamics allowed to differentiate the tested strains into fast-adherent (isolates 007, 008, 025, 042 and *A. acidoterrestris* DSM 3922) and slow-adherent species (isolates 009, 024, 040, 041, 055, 056, 057). Figure 2 shows the patterns of biofilm formation by two isolates, representative for each list-

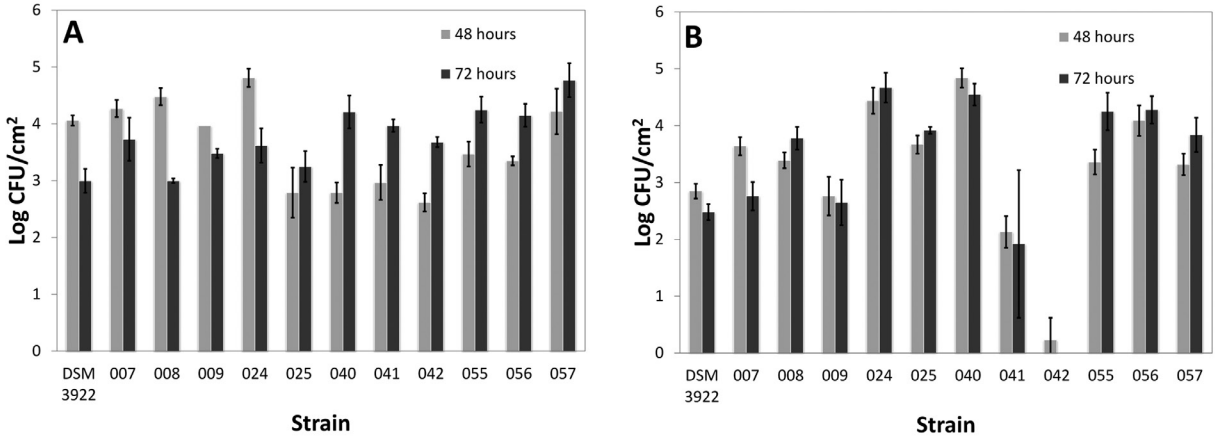

Figure 1. *Alicyclobacillus* sp. biofilm formation within 48-72 hours of incubation
(**A**) culture with agitation; (**B**) culture without agitation)

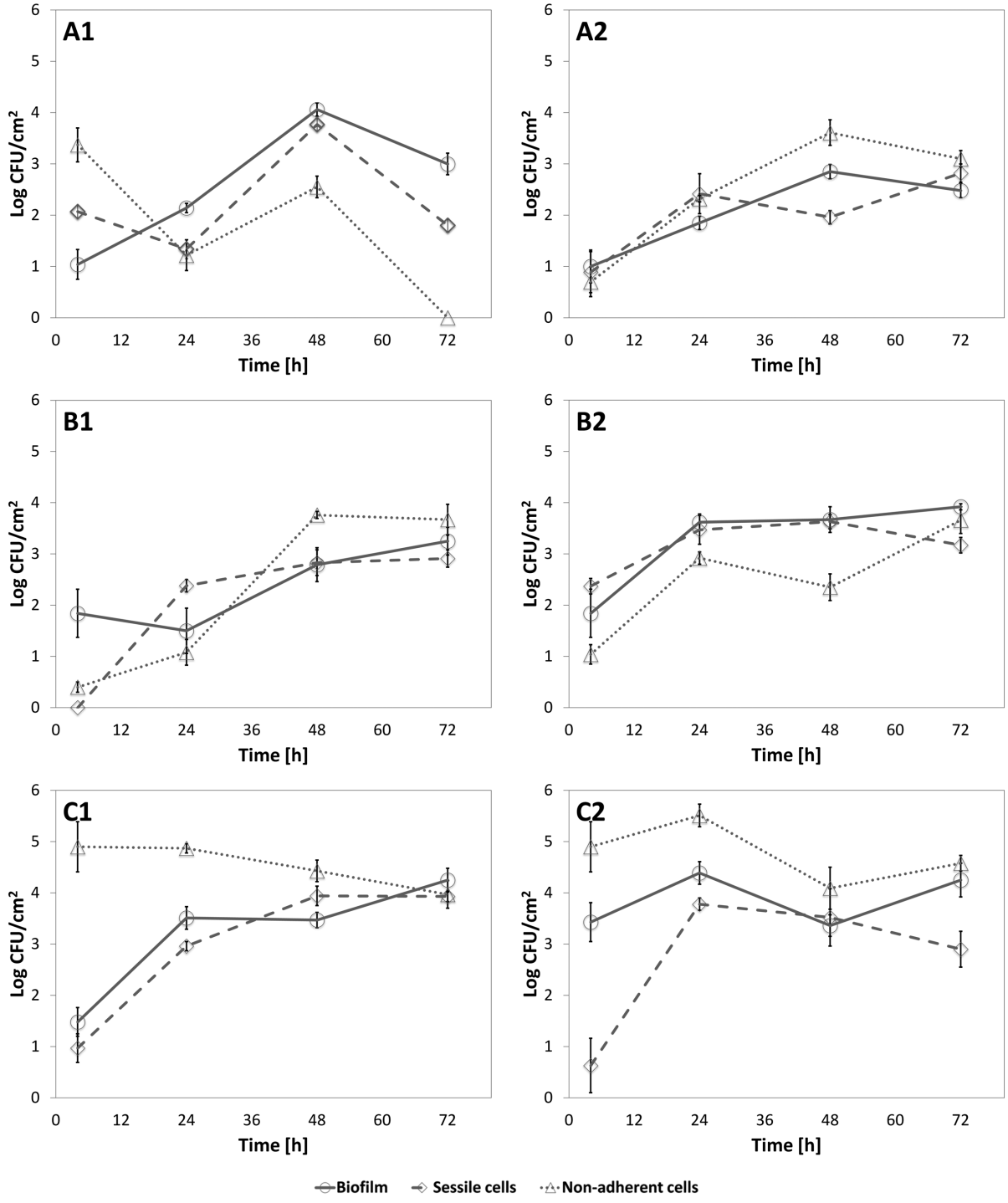

Figure 2. ***Alicyclobacillus*** **acidoterrestris DSM 3922**
(**A**) and *Alicyclobacillus* spp. environmental isolates (**B**) isolate 025; (**C**) isolate 055) biofilm formation dynamics in culture with agitation (**1**) and culture without agitation (**2**)

ed category versus the reference strain DSM 3922. The first category strains express the ability to produce sessile cells on a glass surface as soon as 4-hour incubation. The remaining isolates, both, in static and shaken cultures, seemed either not to adhere to a contact surface so quickly or the established biofilm matrix was rather associated with the presence of non-adherent cells (Fig. 2C1, Fig. 2C2). 75% of the tested bacterial strains expressed an intense increase of biofilm biomass during incubation for 24–48 hours. Further incubation seemed to stabilize the structure of biofilm matrix, as well as to liberate the non-adherent cells, however, slight variations between selected conditions were observed. Although *A. acidoterrestris* DSM 3922 was classified as a rather fast-adherent-strain, its behavior in the non-agitated culture was different. Both, for static and shaken conditions, the amount of viable count for biofilm reached a maximum level (4.06 and 2.85 log CFU/cm² respectively) at the second day of incubation (Fig. 2A1, Fig. 2A2). At the third day, these values were lower (approx. 1.0 and 0.4 log CFU/

cm^2 respectively) and the decrease of loosely attached cells was noted. Biofilm is a multilayer structure in which both, sessile cells and non-adherent cells, could be found (Kolwzan *et al*., 2011; Myszka & Czaczyk, 2011). During the reversible phase of biofilm development, the absence of extracellular polymers on the contact surface and fluid flow may prevent planktonic cells from adhering to the surface. The irreversible stage, as well as a continuous increase of biofilm layers, differentiates sessile cells' metabolic activity. The inner composition of biofilm matrix consists of cells in the state of anabiosis, whereas in the external parts of biofilm, metabolically active agglomerates and individual cells enmeshed within EPS matrix are present (Kolwzan *et al*., 2011). In addition, upper layers of biofilm are reported to provide convenient conditions for sporulation of bacilli (Abee *et al*., 2011). The release of outer bacterial cells and the phenomenon of quorum sensing cause metabolic changes of the whole biofilm structure and regulation of metabolic processes (Garrett *et al*., 2008). Therefore, the decrease in viable cells could be identified as the formation of mature biofilm and early liberation of free-swimming cells. The investigated *A. acidoterrestris* strain expressed higher amount of viable count after 48-hour than after 72-hour incubation. The results obtained here suggest that the biofilm matrix formed on the glass surface after 3 days is dominated by metabolically inactive cells.

Adhesive properties and hydrophobic/hydrophilic interactions have been suggested to participate in the biofilm formation process (Garrett *et al*., 2008; Myszka & Czaczyk, 2011). It has been stated that in general, the lower degree of microbial cells' hydrophobicity, the lower the adhesive ability (Van Loosdrecht *et al*., 1987). However, numerous prior studies provide ambiguous data on dependence between microbial attachment and surface hydrophobicity (Cerca *et al*., 2005; Basson *et al*., 2007; Di Bonaventura *et al*., 2007). A study considering adherence of *Flavobacterium johnson*-like isolates indicated that development of a biofilm in culture with agitation is rather not hydrophobic-dependent (Basson *et al*., 2007). Di Bonaventura *et al*. (2008) claims that development of *Listeria monocytogenes* biofilm on a glass surface is not correlated with hydrophobicity level. On the contrary, the results of Cerca *et al*. (2005) showed a vivid correlation for *Staphylococcus epidermidis* clinical isolates' biofilm forming ability on glass surfaces with their hydrophobicity.

The results obtained in this study for *Alicyclobacillus* environmental isolates show no relationship between a degree of cell hydrophobicity and biofilm formation ability on a glass surface. All of the tested *Alicyclobacillus* strains expressed an ability to develop biofilm and the majority of them were assigned as moderately hydrophobic. Four out of eleven isolates were classified as fast-adherent strains, colonizing the glass surface even within the first 4 hours. The study presented here shows the high risk of biofilm formation on the glass packaging surfaces by alicyclobacilli naturally occurring in the primary production environment in Poland. All the tested *Alicyclobacillus* isolates originating from apples, apple trees and garden soil are capable of colonizing the final product's packaging surface. The problem of alicyclobacilli contamination should not be underestimated, as Poland is the largest apple supplier in the European Union and its importance to the EU apple market ranks third, with a 10% of output ahead of France and Greece (AIJN European Fruit Juice Association, 2014). The apple juice contamination with acidophilic thermophilic bacteria can be associated with biofilm formation and its development, not only on the industrial surfaces exposed to microbial colonization, but also on the recyclable glass packaging. Improper sanitization techniques increase the risk of microbial cross-contamination and in consequence cause a decrease in product shelf-life stability.

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