*Folia Biologica et Oecologica* 12: 26–37 (2016)
Acta Universitatis Lodziensis

# Methods for eradication of the biofilms formed by opportunistic pathogens using novel techniques – A review

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**ABSTRACT**

The inconvenient environmental conditions force microorganisms to colonize either abiotic surfaces or animal and plant tissues and, therefore, form more resistant structures – biofilms. The phenomenon of microbial adherence, opportunistic pathogens in particular, is of a great concern. Colonization of medical devices and biofilm formation on their surface, may lead to severe infections mainly in humans with impaired immune system. Although, current research consider various methods for prevention of microbial biofilms formation, still, once a biofilm is formed, its elimination is almost impossible. This study focuses on the overview of novel methods applied for eradication of mature opportunistic pathogens' biofilms. Among various techniques the following: cold plasma, electric field, ultrasounds, ozonated water treatment, phagotherapy, matrix targeting enzymes, bacteriocins, synthetic chemicals and natural origin compounds used for biofilm matrix disruption were briefly described.

**KEY WORDS:** biofilm eradication, *Pseudomonas aeruginosa*, microbial colonization

## Introduction

### Opportunistic pathogens and the risk they carry

According to a definition, opportunistic pathogens are the organisms which are able to cause disease only when the host's resistance is impaired by other diseases, genetic defects, medical procedures, drugs therapies or age (for example AIDS, cystic fibrosis, chemotherapy, immunosuppression). They are not highly virulent in contrary to true pathogens, that through production of virulence factors may simply evade host defences and harm host tissues (Relman & Falkow 1990).

The conception of opportunistic pathogens is strictly linked to healthcare-associated infections (HAI) as the patients are the most exposed group.

DOI: 10.1515/fobio-2016-0003

According to the European Centre for Disease Prevention and Control (2012), the total number of long term-care-associated infections in EU each year was estimated at 4,3 million. In addition, it was also evaluated that 4,1 million patients acquired the HAI in acute-care facilities. Regarding infection connected with ICU (Intensive Care Unit) the most common among: blood stream infections were caused by coagulase-negative staphylococci, *Enterococcus* spp., *Staphylococcus aureus*; urinary tract infections were *Escherichia coli, Candida* spp., *Enterococcus* spp.; pneumonia cases were *Pseudomonas aeruginosa, S. aureus, E. coli* (Fig.1). All the mentioned microorganisms are supposed to be opportunistic (Annual Epidemiological Report, 2012).

Attempts to remove those microorganisms often fail as they are capable of colonizing medical devices such as catheters, tubes, stents, needles, implants etc. and form a complex structure on these surfaces called biofilm (Zabielska *et al.* 2015).

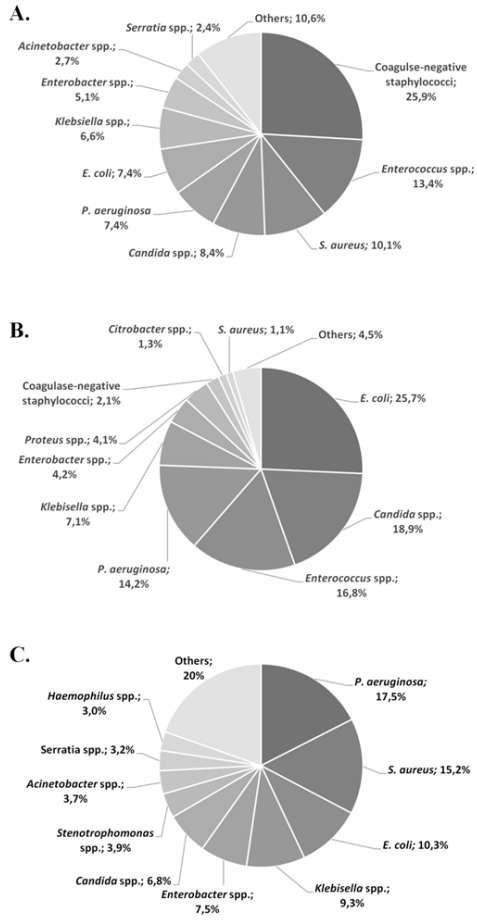


**Figure 1.** Percentage content of microbial infections associated with blood (A), urinary tract (B) and lung (C), (based on Annual Epidemiological Report, 2012).

**Biofilm characteristics and formation**

Biofilms are regarded as dynamic structures of microbial communities of either one or several species enmeshed within extracellular matrix and adhered (classic definition) to biological or abiotic surfaces. Microbial biofilms are also considered as a manner to survive inconvenient environmental condition, as it is reported that cells in a form of the biofilm are more resistant than planktonic ones (Garrett *et al.* 2008). Moreover, researches claim that bacteria embedded in extracellular polymeric substances (EPS) express higher tolerance to antibiotics, disinfectants and are harder to remove from surfaces (Donlan 2001, Furowicz *et al.* 2010, Stewert & Costerton 2001). Biofilms undergo constant changes within their composition, both chemical and biological. External matrix provides suitable conditions for adherence of other microorganisms and, therefore, diversification of biofilms' microbiota.

Formation of biofilm is a complex process which depends on various environmental factors (surface porosity, fluids flow, nutrients availability, etc.) and could be divided into four major steps (Garrett *et al.* 2008). The initial step, in which free-swimming microbial cells attach to the particular biotic or abiotic surfaces, is reversible (Fig.2, A). Planktonic cells can migrate towards the surface of biomaterial by means of physical forces (e.g. van der Waals forces), fluids flow (passive cell transportation) or using their flagella and fimbria (Kolwzan 2011, Haiko & Westerlund-Wikstrom, 2013**)**. At this early stage, single adhered cells do not form a stable structure and, therefore, could be easily removed from the material surface with physical or chemical methods. Whether cells attachment is not affected by any external disruption, the irreversible phase of biofilm formation occurs (Fig. 2, B). The subsequent cell proliferation and production of extracellular polymeric substances (EPS) enables creation of microcolonies enmeshed within biopolymeric matrix (Donlan 2001**)**. The surrounding slime matrix consist of various substances which content differs among microbial species. Nevertheless, major contribution in the EPS composition derives from water and polysaccharides (Czaczyk & Myszka 2007).

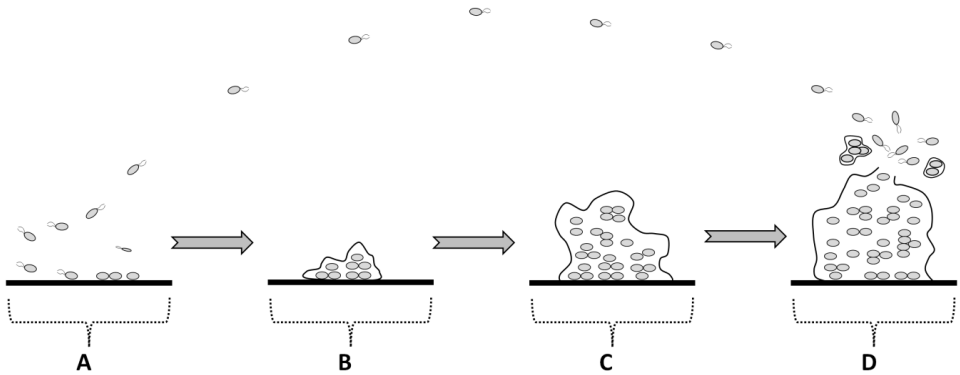


**Figure 2.** Mechanism of biofilm formation: A – single cells attachment and reversible adhesion; B – EPS production, microcolony formation and irreversible adhesion; C – biofilm maturation; D – microbial cells/aggregates dispersal (based on Donlan, 2001; Kolwzan 2011, Maciejewska *et al.* 2016).

The presence of extracellular polymeric substances is pivotal for biofilm functioning. Within the matrix, cells differentiate, form microcolonies, change their metabolism and gradually specialize their functions, therefore, mature biofilms consist of multilayer system (Fig. 2, C). The cells in outer-layer remain active, proliferate and continuously secrete metabolic products. The deeper-laying cells are subjected to limited oxygen and nutrient inflow, thus their metabolism alters toward activation of anaerobic metabolic pathways and inactivation of some enzymes synthesis (Kolwzan 2011). As a result, cells embedded inside the biofilm exhibit different features than planktonic cells.

Biofilm cells differentiation and metabolic activity is associated with signal transduction phenomenon called quorum sensing (QS). QS is a way of communication based on the production of autoinducers (chemical signals) and receptors (proteins receiving signals) which pass from cell to cell (Myszka & Czaczyk 2010). Quorum sensing, thus the cell's communication is, however, facilitated within the biofilm because of microbial density. Accumulation of particular autoinducers 'inform' microbial community about density of their cells and thus helps to maintain proper biofilm regulation. The mechanism of signal transmission is different for Gram-negative and Gram-positive bacteria (Miller & Bassler 2001, Myszka & Czaczyk 2010, Kolwzan 2011). Gram-negative bacteria predominately communicate with acyl homoserine lactone signaling molecules (AHLs). The general structure of AHLs is universal, however, the kind of a substituent incorporated in the α-position is specific for microbial species (Myszka & Czaczyk 2010). On the contrary, Gram-positive bacteria use oligopeptides which are not able to diffuse freely outside the cytoplasmic membrane and should be excreted outside the cell by ATP-dependent transporter proteins (Miller & Bassler 2001, Kolwzan 2011). Although there are specific communication pathways for particular microbial species, there exist a group of universal signal chemicals, called autoinducer-2 (AI-2) molecules, which might enable communication between different microorganisms (Kolwzan 2011).

Signaling pathways of biofilm communities provide proper functioning of this structure. Formation of thick mature biofilm together with accumulation of signal molecules inducers may lead to the disruption of biofilm matrix and the release of single cells or small aggregates. In a consequence, the dispersion of liberated cells enables their propagation among the environment and further colonization of other surfaces (Fig. 2, D). Moreover, quorum sensing might promote particular genes expression which are responsible for antibiotic resistance and anti-drug control (Maciejewska *et al.* 2016). Additionally, the biofilm's EPS coating prevents chemical molecules to enter inside the biofilm structure and act directly onto microbial cells. Therefore, once an irreversible stage of biofilm is achieved, its elimination is hard to obtain or ever impossible. New methods for fighting against biofilms aim to either early stage of biofilm development (reversible adhesion), modification of biomaterials' surfaces or disruption of mature biofilm matrix (Cortez *et al.* 2011, Chen *et al.* 2013).

## Methods for bacterial biofilms eradication

### Physical methods

Since the biofilm structure disposal form surfaces via chemical substances has been well studied, still the easiest way for its elimination seem physical

procedures like e.g. scrapping. However, it is claimed that scrapping is not effective enough due to variety of materials' structures. Another common technique is thermal processing, both in high and low temperatures. Over one-hour exposure in temperature of 95°C significantly reduces the level of microbial biofilms. Similar effects were reported for multiple freezing procedure (Maciejewska *et al*. 2016).

A very promising approach in the process of biofilm eradication seems to be an electromagnetic field. It is reported that Pulsed Electric Fields (PEF) disrupt biofilm matrix of *P. aeruginosa* formed on medical implants (Khan *et al.* 2016). Still the authors suggest that PEF combined with antibiotics may stimulate human immune system and, however, further test involving *in vivo* models should be considered.

The newest researches consider usage of low-temperature (cold) atmospheric pressure plasma for decontamination of surfaces and elimination of bacterial biofilms. A cold plasma treatment with addition of electrospraying against *E. coli* biofilm was studied (Kovalova *et al.* 2016). It was found, that 15-minute exposure to the corona discharge leads to detachment of partial biofilm matrix and the remaining biomass has decreased by 53.6-66.3%. The addition of the water electrospray resulted in more intense *E. coli* biofilm matrix detachment (63.5-70.5% decrease). Similar studies were proceeded by Ziuzina *et al.* (2015), however, not only on *E. coli* biofilm eradication, but also on *P. aeruginosa* virulence testing. The viability of *E. coli* biofilms subjected to a direct and indirect atmospheric cold plasma treatment (ACP) decreased by around 4 log units after 60s exposure. In addition, the metabolic activity of 48-hour *E. coli* biofilm was reduced by about 78% for both direct and indirect ACP exposure. Moreover, the examination of cold plasma treatment applied to *P. aeruginosa* biofilms revealed that ACP acts effectively on two virulence factors of these bacteria – pyocyanin and elastase production. However, the reduction in their concentration did not affect the viability of formed biofilm (Ziuzina *et al.* 2015). On the contrary, the studies conducted by Alkawareek *et al.* (2012) and Ziuzina *et al.* (2014), showed that extended ACP treatment has a significant impact on viability and metabolic activity of *P. aeruginosa* planktonic cells and biofilm matrix.

**Physico-chemical methods**

Ronan *et al.* (2016) have studied the effect of antibiotics (gentamicin or streptomycin) combined with ultrasound and microbubbles (USMB gas-filled microstructures encapsulated by lipid, polymer shell or proteins) treatment against *P. aeruginosa*. Application of USMB, gentamicin or streptomycin alone did not affect the biofilm structure in a great extent. The ultrasounds and microbubbles injection followed by the exposure to antibiotics, resulted in changes in *P. aeruginosa* biofilm matrix and significantly reduced its respiration rate.

The potential anti-biofilm activity was observed for ozonated water as well. The research conducted by Bialoszewski *et al.* (2011) indicates that even 30s exposure of *S. aureus* biofilm to freshly ozonated water results in significant reduction of cells viability. On the contrary, *P. aeruginosa* early stage biofilm expressed higher tolerance, however, mature biofilms (48 and 72-hour biofilms) appeared to be more susceptible to ozonated water. In different study, Hanley-Onken & Cohen (2013) have tested the impact of ozonated water sterilization protocol

against *E. coli* biofilm formed on the stainless steel surface. It was observed that this treatment provides effective biofilm removal and can be used as alternative method for surface sterilization.

Another alternative seems to be photodynamic therapy (PDT) which involves usage of a specific photoactive dye and its activation after an exposure to particular light wavelength (Konopka & Goslinski 2007, Maciejewska 2016). PDT was found to be appropriate as antimicrobial therapy against both drug-resistant microorganisms and biofilms (Hamblin & Hasan 2004, Konopka & Goslinski 2007). Biel *et al.* (2011) reported, that antimicrobial photodynamic therapy tested *in vitro* is effective against planktonic cells and biofilm of *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA). The reduction of both bacteria reached 99.9% after a single treatment. Similar results for both planktonic cells and biofilm were obtained by Street *et al.* (2008). The treatment of free-swimming *P. aeruginosa* cells by means of photodynamic disinfection resulted in more than 7 log units reduction in cell number, whereas 24-hour biofilm was eradicated in 99.0% and 99.9% for single and double exposure respectively.

**Chemical compounds**

Bacteria in biofilm matrix are reported to be less sensitive than planktonic forms towards variety of chemical antimicrobials such as antibiotics, disinfectants and their minimal inhibitory concentration (MICs) are even thousands times higher for biofilm. Many mechanisms are considered to be responsible for biofilm resistance to chemicals. Exopolysaccharides seem to be the main reason as they limit diffusion in the biofilm interior and increase the number of free functional groups. Additionally, slow antimicrobials penetration into further biofilm layers may result in their inactivation by microbial enzymes or removal via efflux pumps. Also the presence of super-resistant cells in deeper layers of biofilm, due to their lack of metabolic activity, weakens the effect of antimicrobials (Sen *et al.* 2015, Kolwzan 2011, Myszka & Czaczyk 2007).

Kwiecinska-Pirog *et al.* (2016) have tested the impact of ciprofloxacin on biofilm formation by *Proteus mirabilis* and *Proteus vulgaris* clinical strains. Ciprofloxacin belongs to 2$^{nd}$ generation quinolones and is considered as the strongest among them. They proved that ciprofloxacin at concentration of 0.06 µg/ml may have been efficient against some strains (reduction over 50%), especially against *P. vulgaris*.

Combination of gentamicin and L-arginine against *S. aureus, E. coli* and *P. aeruginosa* single-strain biofilms were examined by Lebeaux *et al.* (2014). It was found that the addition of L-arginine increased bacteria susceptibility to gentamicin and led to almost complete biofilm eradication at the gentamicin concentration of 200×MIC.

In the research presented by Rosenblatt *et al.* (2015) the synergistic effect of caprilic acid and glyceryl trinitrate (GTN) against MRSA, MRSE (methicillin-resistant *Staphylococcus epidermidis*) and multidrug-resistant *P. aeruginosa* was evaluated. The combination of 0.05% caprylic acid, 0.04% GTN and 5.0% dextrose was very efficient and the biofilm reduction on silicone discs was close to 100% after 2-hour exposure.

Among the recent research an approach of Qu *et al.* (2016) using norspermidine (polyamine) to eradicate *P. aeruginosa* biofilm is noteworthy. The results indicate that norspermidine at concentration of 10 mmol/L can either

prevent from microbial cell attachment to surfaces or disassemble 24-hour mature biofilm with a great efficiency (even 80-90%). This substance also decreases quorum sensing genes expression, pyocyanin production and enzymes activity (elastase, protease).

The other method involves achievements of nanotechnology is usage of nano-penicillin G (Fernandes *et al.* 2016). They obtained nano/micro-sized, oil-filled, surfactant-containing spheres which were able to interact with the membrane of Gram-negative bacteria. Just the presence of surfactant together with penicillin G is crucial for efficient penetration. After *P. aeruginosa* and *E. coli* biofilm contact with nano-penicillin G, they quantified the amount of viable bacteria within biofilms. It was reported that *P. aeruginosa* was more sensitive to the nano-antibiotic than *E. coli*. Similarly penicillin G was used in solution which, in contrast to nano-penicillin G, appeared to be not effective at all against *P. aeruginosa* and induced a 0.8 log CFU/ml reduction of *E. coli* biofilms.

Nanoparticles were also used by Ahmed *et al*. (2016). They treated *Klebsiella penumoniae* biofilm with gold nanoparticles conjugated with chlorchexidine (Au-CHX). A significant biofilm disruption of the tested isolates for Au-CHX at concentration of 100 µM was achieved, whilst non conjugated chlorchexidine even at the concentration of 2 mM was not effective. It was suggested that nanoparticles might have contacted with hard-to-reach bacteria in internal layers of biofilm through water channels formed within biofilm structure.

**Natural compounds and phages**

Currently researchers express a great interest in the use of natural origin substances e.g. essential oils and their constituents. Due to their unique composition and action simultaneously focusing on different targets in a cell, plant derivatives remain effective antimicrobials. Moreover, their usage in combination with antibiotics may exude synergistic effects. The effect of natural substances on microorganisms is multidirectional and includes, inter alia, β-lactamase inhibition, bacterial efflux pump inhibition, cell wall and membrane disturbances and anti-quorum sensing activity (Yap *et al.* 2014).

Anti-biofilm activity of *Mentha pulegium* (Pennyroyal) against multidrug-resistant *Acinetobacter baumanii* was reported by Tutar *et al.* (2016). *M. pulegium* essential oil expressed a strong antimicrobial activity and was able to eradicate biofilm even at ½ MIC concentration. The best results were obtained for this oil at MIC concentration and the reduction in biofilm formation reached 80-90%. Biofilm metabolic activity was also remarkably inhibited at the 2.5 µl/ml essential oil concentration.

The initial attempts involving *S. aureus* biofilm formation and control on stainless steel by component of oregano and thyme essential oil, carvacrol, were proceeded by Knowles & Roller (2001). Combining carvacrol, eugenol and mild micellar surfactants successfully inhibited the growth of *E. coli* O157:H7 and *Listeria monocytogenes* (Perez-Conesa *et al.* 2006). The approach of Yadav *et al.* (2015) based on the effect of eugenol against *S. aureus* was also examined. Eugenol is a major component of clove oil with wide application in food and cosmetic industries due to its antimicrobial, antioxidant, anti-inflammatory, anticarminative and antispasmodic activity. The biomasses of established biofilms of MRSA and MSSA (methicilin-sensitive *Staphylococcus aureus*) were

significantly decreased and their eradication reached the level of 80-90% (0.08% eugenol solution – 2×MIC). The obtained results indicated that eugenol anti-biofilm activity may be due to the disruption of the cell-to-cell connections and cell lysis.

Some of natural compounds express high cytotoxicity, e.g. tea tree oil. Despite good antimicrobial activity *in vitro* their application *in vivo* very often is impossible, since effective concentration is cytotoxic for eukaryotic cells (Hammer *et al.* 2006).

The effect of green tea compound epigallocatechin-3-gallate (EGCg) against *Strenotrophomonas maltophilia* biofilm was evaluated by Vidigal *et al.* (2014). 24-hour and 7-day biofilms after 24-hour exposure to the EGCg were decreased in comparison to untreated biofilms. It is assumed that ECG is capable of binding and damaging bacterial membranes. The antibiofilm effect of green tea was not so spectacular, however it consumed as a beverage or inhaled as a green tea extract solution may serve as a safe agent for intestinal or upper respiratory tract biofilm inhibitor, respectively.

Different group of natural substances are biosurfactants, surface-active substances produced by microorganisms with anti-adhesive and biofilm disruption capabilities.

A novel approach was presented by Diaz De Rienzo *et al.* (2016) who used rhamnolipids and combination of rhamnolipids and caprylic acid against *P. aeruginosa* biofilm. The highest impact on mature biofilm was observed for the mixture of rhamnolipids and caprylic acid (biofilm reduction over 60%). It was found that rhamnolipids may interfere with cell-to-cell interactions and cell-substratum interactions as well.

Nowadays, a particular interest should be paid to novel biological methods in treatment of bacterial biofilms. Apart from the natural substances like plant metabolites or essential oils components, researches considered biofilm eradication with matrix targeting enzymes (Thallinger *et al.* 2013). The enzymes applied cause degradation of biofilm matrix by disruption of extracellular polymeric substances, thus eDNA, proteins and polysaccharides (Chen *et al.* 2013). *In vitro* studies showed that staphylococcal and enterococcal biofilms might be disrupted by N-acetyl-D-glucosamine-1-phosphate acetyl transferase. Similarly, biofilm formed by *S. aureus* was dispersed when treated with proteinase K or trypsin, whereas *S. epidermidis* biofilm matrix was disrupted after dispersin B application (Kaplan *et al.* 2004, Chaignon *et al.* 2007).

Treatment of biofilms with natural microbial substances, bacteriocins, seems to be promising as well. Bacteriocins are considered as protein substances excreted by both Gram-positive and Gram-negative bacteria which aim to inhibit or kill other microorganisms. The effect of three bacteriocins (nisin A, lacticin Q, and nukacin ISK-1) against MRSA was evaluated (Okuda *et al.* 2013). Among three tested substances, bactericidal ability on *S. aureus* biofilms was observed only for nisin A and lacticin Q.

An emerging interest could be find in biofilms elimination by usage of lytic bacteriophages (Carson *et al.* 2010). Great variety of phages has been reported to encode enzymes capable of EPS degradation (Hughes *et al.* 1998). Sharp *et al.* (2010) has described the ability of phages to penetrate through the EPS layer and infect *P. aeruginosa* cells with their polysaccharide lytic enzymes. On the other hand, Carson *et al.* (2010) have studied the effect of bacteriophages on *P.*

*mirabilis* and *E. coli* established biofilms. It was found that phage treatment has reduced the biofilm populations by almost four log units. Further study on biofilms formed on the surface of catheters previously impregnated with hydrogel and exposed to lytic bacteriophages (*E. coli* T4 phage and coli-proteic bacteriophage) showed almost 90% extinction in both *E.coli* and *P. mirabilis* biofilms (Carson *et al.* 2010). The research conducted by Nouraldin *et al.* (2015) concerning concurrent phages and antibiotics application suggests that both planktonic cells and *P. aeruginosa* biofilms are less susceptible when using antibiotics or phages alone. The antibiotic-phage combination expressed a synergistic effect in *P. aeruginosa* biofilm eradication.

## Conclusion

Microbial biofilm is a structure which constantly surprises researchers with its complexity and the mechanisms of development. As the resistance of microorganisms in biofilm can be extremely high, it is crucial to find an effective way to stop the process of biofilm formation or once the biofilm is established, to remove it.

Elimination of biofilm is significant in the clinical environment as opportunistic pathogens colonizing medical equipment may pose a threat for patients with impaired immune system, leading to serious diseases and consequently to death. What is more, it has to be considered that the biofilms may develop on biotic surfaces as well, such as pulmonary epithelium.

The most promising therapies for biofilms eradication seem to be combining gold nanoparticles with antibiotics or antibiotic in the form of nanoparticles, which are able to penetrate deeper layers of biofilm and destroy its internal structure.

Also natural origin substances deserve to be highlighted. Except their ability to eradicate biofilm with a great efficiency there was no increase in microbial resistance after prolonged contact with these specific antimicrobials. Moreover, such compounds may be usually used as food-additives, cosmetic compounds and pharmaceutical products.

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**Streszczenie**

W niekorzystnych warunkach środowiska, mikroorganizmy zasiedlają zarówno powierzchnie abiotyczne, jak i biotyczne takie jak tkanki zwierzęce czy roślinne, tworząc struktury biofilmu charakteryzujące się wysoką opornością. Adhezja mikroorganizmów, szczególnie patogenów oportunistycznych, niesie niebezpieczeństwo zasiedlania materiałów medycznych, co może doprowadzić do infekcji u osób z obniżoną odpornością. Chociaż dotychczasowe badania wskazują różne metody zapobiegania tworzeniu biofilmu, jego całkowita eliminacja ze środowiska jest nadal niemożliwa. Przedstawione opracowanie stanowi przegląd

nowoczesnych metod usuwania dojrzałego biofilmu tworzonego przez patogeny oportunistyczne. Spośród wielu metod opisano m.in. zastosowanie: zimnej plazmy, ultradźwięków, pola elektrycznego, ozonowania wody, terapii fagowej, enzymów działających bezpośrednio na macierz biofilmu, bakteriocyn, środków chemicznych syntetycznych oraz pochodzenia naturalnego.