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4 The current knowledge on the application ofanti-biofilm enzymes in 5 the food industry

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Abstract

Biofilms are encountered on nearly all wet surfaces, with their development being often unwanted due to the serious problems they can cause in different fields, including in the food sector. They are recognized as the preferential microbial lifestyle due to the numerous advantages offered by them for the embedded cells. Biofilm cells are highly resistant to stress conditions, particularly to antimicrobials, since their complex and compact structure hampers the penetration of antimicrobials and the access to the deep positioned cells. The increased resistance to the currently employed control strategies emphasizes the urgent need of new alternative and/or complementary eradication approaches. To this direction, the use of enzymes is an interesting alternative anti-biofilm approach due to their capability to degrade crucial components of the biofilm matrix, cause cell lysis, promote biofilm disruption and interrupt the cell-to-cell signalling events governing biofilm formation and maintenance. This review provides an overview of the enzymes used for biofilm control, their targets and examples of effective applications.

Keywords: Alternative approaches; Extracellular polymeric matrix; Mode of action; Removal; Resistance.

Abbreviations

AHLs	Acyl homoserine lactones
AI-2	Autoinducer-2
AIPs	Autoinducing peptides
AIs	Autoinducers
DNase	Deoxyribonuclease
eDNA	Extracellular DNA
EPS	Extracellular polymeric
QS	substances Quorum-sensing

SS Stainless

US steel

1 Ultrasounds

2 ## 1. Introduction

3 Biofilms are microbial communities attached to either biotic or abiotic surfaces and
4 embedded in a self-produced hydrated polymeric matrix (Cos et al., 2010; Costerton
5 et al., 1995; Simões, 2011; Stoodley et al., 2002). This sessile state represents
6 an outstanding survival strategy for microorganisms, since it protects them against
7 various environmental stresses (e.g. starvation, dehydration) and antimicrobial
8 agents (e.g. antibiotics and biocides) (Costerton et al., 1995; Mah & O'Toole,
9 2001). Although biofilm formation may play an important advantageous role in
10 many processes (e.g. biodegradation of environmental pollutants, plant growth
11 promotion, maintenance of the microbial balance within the human body), it can also
12 cause significant problems in clinical setting and several industries (Bridier et al.,
13 2015; Donlan, 2002; Giaouris et al., 2014; Percival et al., 2011). In fact, biofilms
14 are responsible for persistent human infections, dissemination of pathogens,
15 product contamination, obstruction and corrosion of metallic pipes, decrease of
16 heat transfer efficiency, increase of fluid frictional resistance and other
17 equipment damages, which represent a significant economic and public health
18 concern (Beech, 2004; Cloete et al., 1998; Gilbert et al., 2003; Shi & Zhu, 2009).
19 The biofilm resistance and the consequent failure of the conventional methods to
20 eradicate biofilm-enclosed microorganisms can be explained by: (i) the
21 physicochemical diffusion barrier generated by the presence of an extracellular
22 polymeric matrix; (ii) an altered microbial metabolic state (reduced growth rate/dormant
23 state) in part due to nutrient/oxygen limitation; (iii) the expression of specific
24 resistance genes; and (iv) the differentiation of cells into phenotypic variants less
25 susceptible to treatments (e.g. presence of persister cells) (Anderson & O'Toole,
26 2008; Gilbert et al., 2003; Stewart, 2002).

27 In the food industry, aggressive chemicals, such as sodium hydroxide or
28 sodium hypochlorite, together with clean-in-place techniques are often used to
29 mitigate undesirable biofilm effects. However, such approaches are not always
effective for biofilm control, particularly with respect to the inactivation of the inner
cell layers of

these aggregates and their removal from the surfaces. At the same time, the chemicals used for biofilm control can corrode materials and machinery, endanger users and negatively impact the environment (Gilbert et al., 2003). Among the newly developed biofilm prevention and control approaches are the ones focusing on the intrinsic cellular processes involved in biofilm establishment and maturation, such as motility, cell-to-cell aggregation, production of extracellular polymeric substances (EPS) and intercellular communication (quorum sensing, QS) (Cegelski et al., 2009; Huang & Stewart, 1999; Landini et al., 2010). Therefore, a relevant strategy for removing biofilms from industrial systems is to employ enzymes. Indeed, these have been used for the treatment of biofilms formed in food areas (Anand et al., 2014; Lequette et al., 2010).

2. Anti-biofilm enzymes

Enzymes are natural catalysts capable of accelerating chemical reactions without being consumed (Shanmugam & Sathishkumar, 2009). Undoubtedly, the cellular metabolism depends on these proteins and even minor molecular modifications can have vital metabolic consequences, affecting the complexity of the network of chemical reactions (Cabral et al., 2003). Several factors can interfere with the activity and specificity of enzymes, such as temperature, pH, substrate, presence and/or absence of activators, co-factors or inhibitors (Cabral et al., 2003; Copeland, 2000). The possible applications of these biological molecules are endless, including their use in the industries of foods and beverages, detergents, drugs, textiles, pulp, paper and animal feed (Bajpai, 1999; Kirk et al., 2002). Enzymes can be classified in six main classes: i) oxidoreductases (e.g. alcohol dehydrogenase, glucose oxidase, heme oxygenase, catalase, dihydrofolate reductase, phenylalanine hydroxylase, etc) that catalyse redox reactions and transfer oxygen or hydrogen atoms; ii) transferases (e.g. lipid kinase, transaldolase, phosphomutase, acyl-, methyl-, glucosyl-, phosphoryl-, transferase, etc) that allow the transfer of an atom or a group of atoms from one molecule to another; iii) hydrolases (e.g. serine protease, pectinesterase, glycosylase, pyrophosphatase, aminopeptidase, oligoribonuclease, etc) that catalyse hydrolytic reactions; iv) lyases (e.g. pyruvate decarboxylase, hydratase, aldolase, synthase, etc) that catalyse reactions by removing an atom or a group of atoms; v) isomerases (e.g. isomerase, epimerase and racemase) that catalyse reactions of rearrangement in a molecule; and vi) ligases or synthetases (e.g.

synthetase and carboxylase) thatcan join two molecules together with a covalent bond (Aehle, 2004; Cabral et al., 2003; Shen & Chou, 2007).

The use of enzymes as anti-biofilm agents has increased in recent years (Taraszkiewicz et al., 2013; Thallinger et al., 2013) since this use has been successful in biofilm removal from industrial surfaces. Several applications have been described (Table 1) in an effort to reduce the problems associated to the presence of biofilms and to substitute the harmful and ineffective chemical biocides, thereby providing a greener alternative (Cortés et al., 2011; Srey et al., 2013). The application of enzymes for the cleaning of the food contact surfaces is approved by the regulatory agencies (Schmidt, 1997) and there is no evidence related to the interference of the enzymatic treatments with the food quality. Indeed, provided the surfaces are properly rinsed there is no possibility of food contamination or the risk for an enzyme to be considered an additional illegal additive (Troller, 1993).

2.1. Mode of action

The target of biofilm-disrupting enzymes is usually the EPS matrix surrounding the cells (Lequette et al., 2010; Xavier et al., 2005). However, their mode of action can greatly vary. Enzymes can: i) attack directly the biofilm components and degrade them; ii) induce cellular lysis; iii) interfere with the QS system; iv) or even catalyse the formation of antimicrobials (Augustin et al., 2004; Cordeiro & Werner, 2011; Donlan, 2002; Simões et al., 2010; Thallinger et al., 2013). The action of enzymes is intrinsically related to the decrease of biofilm physical integrity, degrading matrix molecules into monomers that can be transported through the cell and further metabolized (Molobela et al., 2010). As enzymes can act on the biofilm EPS, the structural components of this matrix should be ideally identified before any enzymatic application (Molobela et al., 2010). Carbohydrates, polysaccharides, proteins (frequently exhibiting amyloid-like properties), glycoproteins, lipids, phospholipids, glycolipids, and nucleic acids are usually identified as components of the EPS matrix (Branda et al., 2005; Flemming & Wingender, 2010; Hobley et al., 2015). The matrix composition and architecture is dependent on a number of extrinsic factors, including fluctuations in nutrient and gaseous levels and fluid shear (Simões et al. 2010). Moreover, a range of complex enzymatic and regulatory activities can be found within the matrix (Allison, 2003; Sutherland, 1999).

By using enzymes, the in-use biocides can be either replaced or their concentration can be significantly reduced since the enzymatic action on the EPS matrix favours the access of the chemicals to the cells (Cortés et al., 2011; Lequette et al., 2010; Srey et al., 2013). Given that biofilms can have heterogeneous composition, diverse types of enzymes are required to combat them and usually a mixture of enzymes should be applied, or combined with complementary treatments (Augustin et al., 2004; Kumar & Anand, 1998; Thallinger et al., 2013). There are four types of enzymes of particular interest for biofilm removal: anti-QS enzymes, oxidative enzymes (Thallinger et al., 2013), polysaccharide-degrading enzymes and proteolytic enzymes, (Johansen et al., 1997; Thallinger et al., 2013). These four types of enzymes belong to three of the main classes mentioned before: hydrolases, oxidoreductases and lyases (Figure 1).

2.2. Anti-quorum sensing enzymes

The close proximity of cells in biofilms and the spatio-chemical conditions enables bacterial coexistence and the retaining matrix provides optimal conditions for QS phenomenon (Giaouris et al., 2015; Li & Tian, 2012). QS is a form of intercellular communication used by many species of bacteria in response to an increase in cell density. This complex gene regulatory system relies on the production, release and detection of small signaling molecules called autoinducers (AIs) (LaSarre & Federle, 2013). Several chemical classes of microbial derived signaling molecules are already identified, with the most commonly studied ones belonging to one of the following three categories: acyl homoserine lactones (AHLs), autoinducing peptides (AIPs) and autoinducer-2 (AI-2) (Miller & Bassler, 2001). QS systems are comprised of three components: the AI, the gene coding for the AI synthase protein and the gene coding for the response regulator protein. Therefore, whatever the efforts employed to disrupt the QS phenomenon, all strategies are based on the inhibition of one of these mechanisms (Kalia, 2013). Given the typical involvement of QS in biofilm development and maintenance, anti-QS enzymes could be used (Lazar, 2011). Examples of such enzymes are N-acyl homoserine lactonases and acylases. Lactonases are anti-QS enzymes that hydrolyse the bond in the homoserine ring, avoiding the binding of homoserine lactones (AHLs) to transcriptional regulators (Thallinger et al., 2013). Kiran et al. (2011) used a lactonase in their studies and achieved 69% to 77% biofilm reduction of *Pseudomonas aeruginosa*, as well as a decrease in the production of virulence factors. Kim et al.

(2013) used acylase I as it is able to cleave QS molecules. This enzyme (at 100 μg/mL) was only able to remove 9.0% of the cells present in a reverse osmosis membrane (Kim et al., 2013). In another study, Pei and Lamas-Samanamud (2014) constructed an engineered T7 bacteriophage expressing a lactonase with broad-range activity for QS inhibition. The addition of the engineered phage to mixed-species biofilms containing *P. aeruginosa* and *Escherichia coli* resulted in inhibition of biofilm formation on polyvinyl chloride microtiter plates.

2.3. Oxidative enzymes

Enzymatic treatments can also target the extracellular DNA (eDNA) encountered in the biofilm matrix (Hall-Stoodley et al., 2008; Moscoso et al., 2006; Okshevsky & Meyer, 2015; Thomas et al., 2008). Indeed, its enzymatic degradation can prevent, disperse, or sensitize biofilms to antimicrobials (Okshevsky et al., 2015). Thomas et al. (2008) showed that treatments with deoxyribonuclease (DNase) reduced biofilm accumulation of *Enterococcus faecalis*. DNase was also used to control *Streptococcus pneumoniae* biofilms (Hall-Stoodley et al., 2008; Moscoso et al., 2006). Hall-Stoodley et al. (2008) observed a decrease in the biofilm thickness higher than 85%. Nguyen and Burrows (2014) studied the attachment of *Listeria monocytogenes* to polystyrene surfaces and verified that the addition of DNase at the beginning of biofilm formation enabled the reduction of *L. monocytogenes* attachment by 50%.

2.4. Polysaccharide-degrading enzymes

Polysaccharide-degrading anti-biofilm enzymes are composed by amylase, alginate lyase, cellulase and lysozyme (Lequette et al., 2010; Thallinger et al., 2013). Loiselle and Anderson (2003) reported that the enzyme cellulase inhibits *P. aeruginosa* biofilm formation. The effect of cellulase in breaking down EPS was supported by the decrease of the apparent molecular weight of these substances and by the increase in reducing sugars production (Loiselle & Anderson, 2003). In fact, the enzymatic specificity is dependent on the microorganism as it was shown by Craigen et al. (2011). In their study α-amylase was only capable of reducing *Staphylococcus aureus* biofilm by 79%, while it was not efficient in removing *Staphylococcus epidermidis* biofilms (Craigen et al., 2011). Brindle et al. (2011) used dispersinB since it hydrolyses a polysaccharide

1 excreted by *S. epidermidis* and is responsible for the biofilm structure. This enzyme was
2 applied at 40 ppm on biofilms of *S. epidermidis* on glass surfaces allowing a
3 40% biofilm removal (Brindle et al., 2011).

4

5 **2.5. Proteolytic enzymes**

6 A class of proteolytic enzymes are proteases. These enzymes were shown to hydrolyse
7 proteins in pipelines (Augustin et al., 2004). Molobela et al. (2010) tested
8 several enzymes and described Savinase® as one of the most efficient enzymatic
9 preparation in removing *Pseudomonas fluorescens* biofilms from glass wool. The
10 authors also concluded that the biofilm structural composition needs to be taken into
11 account since they found that amylases were less effective than proteases in degrading
12 *P. fluorescens* biofilms (Molobela et al., 2010). Likewise, Huang et al. (2014) had the
13 same results, i.e. proteases were more efficient in removing biofilms. Indeed, Leroy
14 et al. (2008) used Savinase® to avoid *Pseudoalteromonas* sp. adhesion and biofilm
15 formation, causing complete biofilm removal. Augustin et al. (2004) used
16 different enzymatic agents (Pandion, Resinase, Spezyme and Paradigm,
17 individually applied), for 30 min, and achieved a 4 log reduction of *P. aeruginosa*
18 population. Orgaz et al. (2007) applied Pronase to *P. fluorescens* biofilms formed on
19 borosilicate glass surfaces and were able to remove 30% of the biofilm.

20 Bacteriophages (or phages) are viruses that infect bacteria and ultimately cause cell lysis
21 (Donlan, 2009; Fischetti, 2005). For this reason they can also be used as an anti-biofilm
22 strategy (Sillankorva & Azeredo, 2014; Simões et al., 2010). The cell lysis is caused by
23 lysins that are produced by the phages (Fischetti, 2005). Furthermore, the phages
24 can also produce polysaccharide depolymerases that are able to disrupt the EPS
25 matrix (Donlan, 2009; Hughes et al., 1998). Sharma et al. (2005) used
26 bacteriophages and reduced biofilms of *E. coli* O157:H7 by 2.8 log CFU per
27 stainless steel (SS) coupon. Gutiérrez et al. (2014) were able to reduce *S. aureus*
28 (isolated from a food environment) biofilm by 1-3 log units per well of polystyrene
29 microtiter plates using endolysin - LysH5 that induced cell lysis (Borysowski et al.,
30 2006).

31 Several authors (Fischetti, 2005; Lu & Collins, 2007; Tait et al., 2002) already
32 used synthetic biology to increase bacteriophages action, by engineering them to
produce biofilm-disrupting enzymes. These bacteriophages can simultaneously
and more

effectively cause both cell lysis and matrix disruption (Lu & Collins, 2007). For instance, Lu and Collins (2007) produced a bacteriophage that was able to produce a biofilm degrading enzyme that reduced *E. coli* biofilm by 99.997%, a capability which was about two orders of magnitude better than that of the nonenzymatic phage.

2.6. Combination of enzymes

The use of enzymatic combinations has already been demonstrated as an effective anti-biofilm strategy (Johansen et al., 1997; Orgaz et al., 2007; Yamasaki et al., 2005). Johansen et al. (1997) firstly tested the activity of individual oxidoreductases and polysaccharide-hydrolysing enzymes and found a bactericidal effect and a biofilm removing capacity, respectively. However, when both enzymes were combined, biofilm removal and cell inactivation were simultaneously observed (Johansen et al., 1997). Kim et al. (2013) mixed acylase I (100 µg/mL) with proteinase K (5 µg/mL) and were able to remove 33.7% of the cells present in a reverse osmosis membrane. Orgaz et al. (2007) applied cellulase followed by Pronase to *P. fluorescens* biofilms formed on borosilicate glass surfaces and were able to remove 94% of the biofilm.

2.7. Combination of enzymes with chemical/physical treatments

The combination of enzymes with other antimicrobial techniques/compounds, such as ultrasounds and biocides, has also been studied and proved to be more efficient than the individual treatments applied alone. Oulahal et al. (2007) combined enzymes (protease, trypsin, amyloglucosidase, papain and lysozyme) with ultrasounds (40 kHz for 10 s) and a chelating agent (ethylene-diamine tetraacetic acid - EDTA) to remove biofilms from stainless steel surfaces. The combination allowed a removal of 75% and 100% of *E. coli* and *S. aureus* biofilms, respectively (Oulahal et al., 2007). Alkawash et al.(2006) achieved complete removal of *P. aeruginosa* biofilms on cellulose fibbers, when applied alginate lyase and gentamycin for 96 hours. Pechaud et al. (2012) observed that the combination of Savinase® with shear stress (2.5 Pa) promoted *P. aeruginosa* biofilm removal by 90%. Whereas only 20% removal was observed when sodium hypochlorite (50 ppm) was used individually. Oulahal-Lagsir et al. (2003) used proteolytic enzymes and ultrasounds (40 kHz for 10 s) to remove *E. coli* biofilms from stainless steel surfaces. In that study, the combination of amyloglucosidase (50 $U.mL^{-1}$) exerted

synergistic action by causing 96% biofilm removal (Oulahal-Lagsir et al., 2003). Lequette et al. (2010) combinedabufferwithananionicsurfactantmixedwithα-amylase and reduced *Bacillus mycoides* biofilm on stainless steel by 2.98 log CFU/cm^2.

Figure 2 shows the biofilm removal action of a mixture of enzymes (Biorem 10) with surfactants (Biorem A1) as proposed by Realco (Belgium) (Figure 2b). Complete biofilm removal was achieved when this cocktail was combined with sodium hypochlorite (Figure 2c).

3. Conclusions

The recent improvements in understanding the mechanisms underlying biofilm formation and resistance have enabled the development of new and more effective anti-biofilm strategies. Successful approaches should promote both microbial killing/inactivation and removal of the attached biomass. Biofilm-disrupting enzymes have the ability to degrade different components of the biofilm matrix, while some of them are also able to interrupt bacterial communication, affecting not only biofilm development but also promoting their eradication. The employment of enzymes is advantageous as they do not impose selective pressure on bacteria and have a green-status. However, taking into account that the composition of the biofilm matrix is complex, and enzymes have a specific character, the use of mixture of enzymes is often required. The current knowledge clearly proposes the application of engineered enzymes (using synthetic biology) and also the combined application of enzymes with other antimicrobial treatments (chemical and/or physical) as valuable approaches for effective biofilm mitigation. Such approaches will certainly reduce the drawbacks related to the high cost of enzymes and the requirements for specific environmental conditions, increasing the interest on their use as anti-biofilm agents.

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8 **References**

9 Aehle, W. (2004). Introduction. In W. Aehle (Ed.), *Enzymes in industry: Products and*
10 *applications* (2nd ed., pp. 1-12). The Netherlands: John Wiley & Sons.
11 Alkawash, M. A., Soothill, J. S., & Schiller, N. L. (2006). Alginate lyase enhances
12 antibiotic killing of mucoid *Pseudomonas aeruginosa* in biofilms. *APMIS,*
13 *114*(2), 131-138.
14 Allison, D. G. (2003). The biofilm matrix. *Biofouling, 19*(2), 139-150.
15 Anand, S., Singh, D., Avadhanula, M., & Marka, S. (2014). Development and Control
16 of Bacterial Biofilms on Dairy Processing Membranes. *Comprehensive Reviews*
17 *in Food Science and Food Safety, 13*(1), 18-33.
18 Anderson, G. G., & O'Toole, G. A. (2008). Innate and induced resistance mechanisms
19 of bacterial biofilms. *Current Topics in Microbiology and Immunology, 322*, 85-
20 105.
21 Augustin, M., Ali-Vehmas, T., & Atroshi, F. (2004). Assessment of enzymatic cleaning
22 agents and disinfectants against bacterial biofilms. *Journal of Pharmacy &*
23 *Pharmaceutical Sciences, 7*(1), 55-64.
24 Bajpai, P. (1999). Application of enzymes in the pulp and paper industry. *Biotechnology*
25 *Progress, 15*(2), 147-157.
26 Beech, I. B. (2004). Corrosion of technical materials in the presence of biofilms -
27 current understanding and state-of-the art methods of study. *International*
28 *Biodeterioration & Biodegradation, 53*(3), 177-183.
29 Borysowski, J., Weber-Dabrowska, B., & Gorski, A. (2006). Bacteriophage endolysins
30 as a novel class of antibacterial agents. *Experimental Biology and Medicine,*
31 *231*(4), 366-377.

Branda, S. S., Vik, S., Friedman, L., & Kolter, R. (2005). Biofilms: the matrix revisited. *Trends in Microbiology, 13*(1), 20-26.

Bridier, A., Sanchez-Vizuete, P., Guilbaud, M., Piard, J. C., Naïtali, M., & Briandet, R. (2015). Biofilm-associated persistence of food-borne pathogens. *Food Microbiology, 45, Part B*, 167-178.

Brindle, E. R., Miller, D. A., & Stewart, P. S. (2011). Hydrodynamic deformation and removal of Staphylococcus epidermidis biofilms treated with urea, chlorhexidine, iron chloride, or DispersinB. *Biotechnology and Bioengineering, 108*(12), 2968-2977.

Cabral, J., Gama, M., & Aires-Barros, M. (2003). Introdução. In J. Cabral, M. Aires-Barros & M. Gama (Eds.), *Engenharia Enzimática* (pp. 1-12). Portugal: Lidel.

Cegelski, L., Pinkner, J. S., Hammer, N. D., Cusumano, C. K., Hung, C. S., Chorell, E., Åberg, V., Walker, J. N., Seed, P. C., Almqvist, F., Chapman, M. R., & Hultgren, S. J. (2009). Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. *Nature Chemical Biology, 5*(12), 913-919.

Cloete, T. E., Jacobs, L., & Brözel, V. S. (1998). The chemical control of biofouling in industrial water systems. *Biodegradation, 9*(1), 23-37.

Copeland, R. A. (2000). A brief history of enzymology. In R. A. Copeland (Ed.), *Enzymes: A Practical Introduction To Structure, Mechanism, And Data Analysis* (2nd ed., pp. 1-10). United States of America: John Wiley & Sons.

Cordeiro, A. L., & Werner, C. (2011). Enzymes for Antifouling Strategies. *Journal of Adhesion Science and Technology, 25*(17), 2317-2344.

Cortés, E., Consuegra, J., Sinisterra, R., & Mendez-Vilas, A. (2011). Biofilm formation, control and novel strategies for eradication (Vol. 2, pp. 896-905): Science Against Microbial Pathogens: Communicating Current Research and Technological Advances.

Cos, P., Toté, K., Horemans, T., & Maes, L. (2010). Biofilms: An extra hurdle for effective antimicrobial therapy. *Current Pharmaceutical Design, 16*(20), 2279-2295.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms. *Annual Review of Microbiology, 49*, 711-745.

Craigen, B., Dashiff, A., & Kadouri, D. E. (2011). The use of commercially available alpha-amylase compounds to inhibit and remove *Staphylococcus aureus* biofilms. *Open Microbiology Journal, 5*, 21-31.

1 Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerging Infectious*
2 *Diseases, 8*(9), 881-890.
3 Donlan, R. M. (2009). Preventing biofilms of clinically relevant organisms using
4 bacteriophage. *Trends in Microbiology, 17*(2), 66-72.
5 Fischetti, V. A. (2005). Bacteriophage lytic enzymes: novel anti-infectives. *Trends in*
6 *Microbiology, 13*(10), 491-496.
7 Flemming, H.-C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews*
8 *Microbiology, 8*(9), 623-633.
9 Giaouris, E., Heir, E., Desvaux, M., Hebraud, M., Moretro, T., Langsrud, S.,
10 Doulgeraki, A., Nychas, G. J., Kacaniova, M., Czaczyk, K., Olmez, H., &
11 Simões, M. (2015). Intra- and inter-species interactions within biofilms of
12 important foodborne bacterial pathogens. *Frontiers in Microbiology, 6*, 841.
13 Giaouris, E., Heir, E., Hébraud, M., Chorianopoulos, N., Langsrud, S., Møretrø, T.,
14 Habimana, O., Desvaux, M., Renier, S., & Nychas, G.-J. (2014). Attachment
15 and biofilm formation by foodborne bacteria in meat processing environments:
16 Causes, implications, role of bacterial interactions and control by alternative
17 novel methods. *Meat Science, 97*(3), 298-309.
18 Gilbert, P., McBain, A. J., & Rickard, A. H. (2003). Formation of microbial biofilm in
19 hygienic situations: A problem of control. *International Biodeterioration and*
20 *Biodegradation, 51*(4), 245-248.
21 Gutiérrez, D., Ruas-Madiedo, P., Martínez, B., Rodríguez, A., & García, P. (2014).
22 Effective removal of Staphylococcal biofilms by the endolysin LysH5. *PLoS*
23 *ONE, 9*(9), e107307.
24 Hall-Stoodley, L., Nistico, L., Sambanthamoorthy, K., Dice, B., Nguyen, D., Mershon,
25 W. J., Johnson, C., Hu, F. Z., Stoodley, P., & Ehrlich, G. D. (2008).
26 Characterization of biofilm matrix, degradation by DNase treatment and
27 evidence of capsule downregulation in *Streptococcus pneumoniae* clinical
28 isolates. *BMC Microbiology, 8*(1), 173.
29 Hobley, L., Harkins, C., MacPhee, C. E., & Stanley-Wall, N. R. (2015). Giving
30 structure to the biofilm matrix: an overview of individual strategies and
31 emerging common themes. *FEMS Microbiology Reviews, 39*(5), 649-669.
32 Huang, C. T., & Stewart, P. S. (1999). Reduction of polysaccharide production in
33 *Pseudomonas aeruginosa* biofilms by bismuth dimercaprol (BisBAL) treatment.
34 *The Journal of Antimicrobial Chemotherapy, 44*(5), 601-605.

1 Huang, H., Ren, H., Ding, L., Geng, J., Xu, K., & Zhang, Y. (2014). Aging biofilm
2 from a full-scale moving bed biofilm reactor: characterization and enzymatic
3 treatment study. *Bioresource Technology, 154*, 122-130.
4 Hughes, K. A., Sutherland, I. W., Clark, J., & Jones, M. V. (1998). Bacteriophage and
5 associated polysaccharide depolymerases - novel tools for study of bacterial
6 biofilms. *Journal of Applied Microbiology, 85*(3), 583-590.
7 Johansen, C., Falholt, P., & Gram, L. (1997). Enzymatic removal and disinfection of
8 bacterial biofilms. *Applied and Environmental Microbiology, 63*(9), 3724-3728.
9 Kalia, V. C. (2013). Quorum sensing inhibitors: an overview. *Biotechnology Advances,*
10 *31*(2), 224-245.
11 Kim, L. H., Kim, S.-J., Kim, C.-M., Shin, M. S., Kook, S., & Kim, I. S. (2013). Effects
12 of enzymatic treatment on the reduction of extracellular polymeric substances
13 (EPS) from biofouled membranes. *Desalination and Water Treatment, 51*(31-
14 33), 6355-6361.
15 Kiran, S., Sharma, P., Harjai, K., & Capalash, N. (2011). Enzymatic quorum quenching
16 increases antibiotic susceptibility of multidrug resistant *Pseudomonas*
17 *aeruginosa*. *Iranian Journal of Microbiology, 3*(1), 1-12.
18 Kirk, O., Borchert, T. V., & Fuglsang, C. C. (2002). Industrial enzyme applications.
19 *Current Opinion in Biotechnology, 13*(4), 345-351.
20 Kumar, C. G., & Anand, S. K. (1998). Significance of microbial biofilms in food
21 industry: A review. *International Journal of Food Microbiology, 42*(1-2), 9-27.
22 Landini, P., Antoniani, D., Burgess, J. G., & Nijland, R. (2010). Molecular mechanisms
23 of compounds affecting bacterial biofilm formation and dispersal. *Applied*
24 *Microbiology and Biotechnology, 86*(3), 813-823.
25 LaSarre, B., & Federle, M. J. (2013). Exploiting quorum sensing to confuse bacterial
26 pathogens. *Microbiology and Molecular Biology Reviews, 77*(1), 73-111.
27 Lazar, V. (2011). Quorum sensing in biofilms - how to destroy the bacterial citadels or
28 their cohesion/power? *Anaerobe, 17*(6), 280-285.
29 Lequette, Y., Boels, G., Clarisse, M., & Faille, C. (2010). Using enzymes to remove
30 biofilms of bacterial isolates sampled in the food-industry. *Biofouling, 26*(4),
31 421-431.
32 Leroy, C., Delbarre, C., Ghillebaert, F., Compere, C., & Combes, D. (2008). Effects of
33 commercial enzymes on the adhesion of a marine biofilm-forming bacterium.
34 *Biofouling, 24*(1), 11-22.

Li, Y.-H., & Tian, X. (2012). Quorum sensing and bacterial social interactions in biofilms. *Sensors, 12*(3), 2519-2538.

Loiselle, M., & Anderson, K. W. (2003). The use of cellulase in inhibiting biofilm formation from organisms commonly found on medical implants. *Biofouling, 19*(2), 77-85.

Lu, T. K., & Collins, J. J. (2007). Dispersing biofilms with engineered enzymatic bacteriophage. *Proceedings of the National Academy of Sciences, 104*(27), 11197-11202.

Mah, T. F., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology, 9*(1), 34-39.

Miller, M. B., & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annual Review of Microbiology, 55*, 165-199.

Molobela, I. P., Cloete, T. E., & Beukes, M. (2010). Protease and amylase enzymes for biofilm removal and degradation of extracellular polymeric substances (EPS) produced by *Pseudomonas fluorescens* bacteria. *African Journal of Microbiology Research, 4*(14), 1515-1524.

Moscoso, M., García, E., & López, R. (2006). Biofilm formation by *Streptococcus pneumoniae*: Role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *Journal of Bacteriology, 188*(22), 7785-7795.

Nguyen, U. T., & Burrows, L. L. (2014). DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms. *International Journal of Food Microbiology, 187*, 26-32.

Okshevsky, M., & Meyer, R. L. (2015). The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. *Critical Reviews in Microbiology, 41*(3), 341-352.

Okshevsky, M., Regina, V. R., & Meyer, R. L. (2015). Extracellular DNA as a target for biofilm control. *Current Opinion in Biotechnology, 33*, 73-80.

Orgaz, B., Neufeld, R. J., & SanJose, C. (2007). Single-step biofilm removal with delayed release encapsulated Pronase mixed with soluble enzymes. *Enzyme and Microbial Technology, 40*(5), 1045-1051.

Oulahal-Lagsir, N., Martial-Gros, A., Bonneau, M., & Blum, L. J. (2003). "*Escherichia coli*-milk" biofilm removal from stainless steel surfaces: synergism between ultrasonic waves and enzymes. *Biofouling, 19*(3), 159-168.

1 Oulahal, N., Martial-Gros, A., Bonneau, M., & Blum, L. J. (2007). Removal of meat
2 biofilms from surfaces by ultrasounds combined with enzymes and/or a
3 chelating agent. *Innovative Food Science and Emerging Technologies, 8*(2),
4 192-196.
5 Pechaud, Y., Marcato-Romain, C. E., Girbal-Neuhauser, E., Queinnec, I., Bessiere, Y.,
6 & Paul, E. (2012). Combining hydrodynamic and enzymatic treatments to
7 improve multi-species thick biofilm removal. *Chemical Engineering Science,*
8 *80*, 109-118.
9 Pei, R., & Lamas-Samanamud, G. R. (2014). Inhibition of biofilm formation by T7
10 bacteriophages producing quorum-quenching enzymes. *Applied and*
11 *Environmental Microbiology, 80*(17), 5340-5348.
12 Percival, S. L., Malic, S., Cruz, H., & Williams, D. W. (2011). Introduction to biofilms.
13 In S. L. K. Percival, Derek C.; Cochrane, Christine A. (Eds.) (Ed.), *Biofilms and*
14 *Veterinary Medicine* (Vol. 6, pp. 41-68): Springer-Verlag Berlin Heidelberg
15 Schmidt, R. H. (1997). *Basic elements of equipment cleaning and sanitizing in food*
16 *processing and handling operations*: University of Florida Cooperative
17 Extension Service, Institute of Food and Agriculture Sciences, EDIS.
18 Shanmugam, S., & Sathishkumar, T. (2009). Enzyme extraction and isolation. In S.
19 Shanmugam & T. Sathishkumar (Eds.), *Enzyme Technology* (pp. 21-27). New
20 Delhi: I. K. International Publishing House.
21 Sharma, M., Ryu, J. H., & Beuchat, L. R. (2005). Inactivation of *Escherichia coli*
22 O157:H7 in biofilm on stainless steel by treatment with an alkaline cleaner and a
23 bacteriophage. *Journal of Applied Microbiology, 99*(3), 449-459.
24 Shen, H.-B., & Chou, K.-C. (2007). EzyPred: A top–down approach for predicting
25 enzyme functional classes and subclasses. *Biochemical and Biophysical*
26 *Research Communications, 364*(1), 53-59.
27 Shi, X., & Zhu, X. (2009). Biofilm formation and food safety in food industries. *Trends*
28 *in Food Science & Technology, 20*(9), 407-413.
29 Sillankorva, S., & Azeredo, J. (2014). Bacteriophage attack as an anti-biofilm strategy.
30 *Methods in Molecular Biology, 1147*, 277-285.
31 Simões, M. (2011). Antimicrobial strategies effective against infectious bacterial
32 biofilms. *Current Medicinal Chemistry, 18*(14), 2129-2145.
33 Simões, M., Simões, L. C., & Vieira, M. J. (2010). A review of current and emergent
34 biofilm control strategies. *LWT - Food Science and Technology, 43*(4), 573-583.

Srey, S., Jahid, I. K., & Ha, S.-D. (2013). Biofilm formation in food industries: A food safety concern. *Food Control, 31*(2), 572-585.

Stewart, P. S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology : IJMM, 292*(2), 107-113.

Stoodley, P., Sauer, K., Davies, D. G., & Costerton, J. W. (2002). Biofilms as complex differentiated communities. *Annual Review of Microbiology, 56*, 187-209.

Sutherland, I. (1999). Polysaccharases in Biofilms — Sources — Action — Consequences! In J. Wingender, T. Neu & H.-C. Flemming (Eds.), *Microbial Extracellular Polymeric Substances* (pp. 201-216): Springer Berlin Heidelberg.

Tait, K., Skillman, L. C., & Sutherland, I. W. (2002). The efficacy of bacteriophage as a method of biofilm eradication. *Biofouling, 18*(4), 305-311.

Taraszkiewicz, A., Fila, G., Grinholc, M., & Nakonieczna, J. (2013). Innovative strategies to overcome biofilm resistance. *BioMed Research International, 2013*, 13.

Thallinger, B., Prasetyo, E. N., Nyanhongo, G. S., & Guebitz, G. M. (2013). Antimicrobial enzymes: An emerging strategy to fight microbes and microbial biofilms. *Biotechnology Journal, 8*(1), 97-109.

Thomas, V. C., Thurlow, L. R., Boyle, D., & Hancock, L. E. (2008). Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. *Journal of Bacteriology, 190*(16), 5690-5698.

Troller, J. A. (1993). Chapter 4 - Cleaning, *Sanitation in Food Processing* (2nd ed., pp. 30-51). London: Academic Press.

Xavier, J. B., Picioreanu, C., Rani, S. A., van Loosdrecht, M. C., & Stewart, P. S. (2005). Biofilm-control strategies based on enzymic disruption of the extracellular polymeric substance matrix - a modelling study. *Microbiology, 151*(Pt 12), 3817-3832.

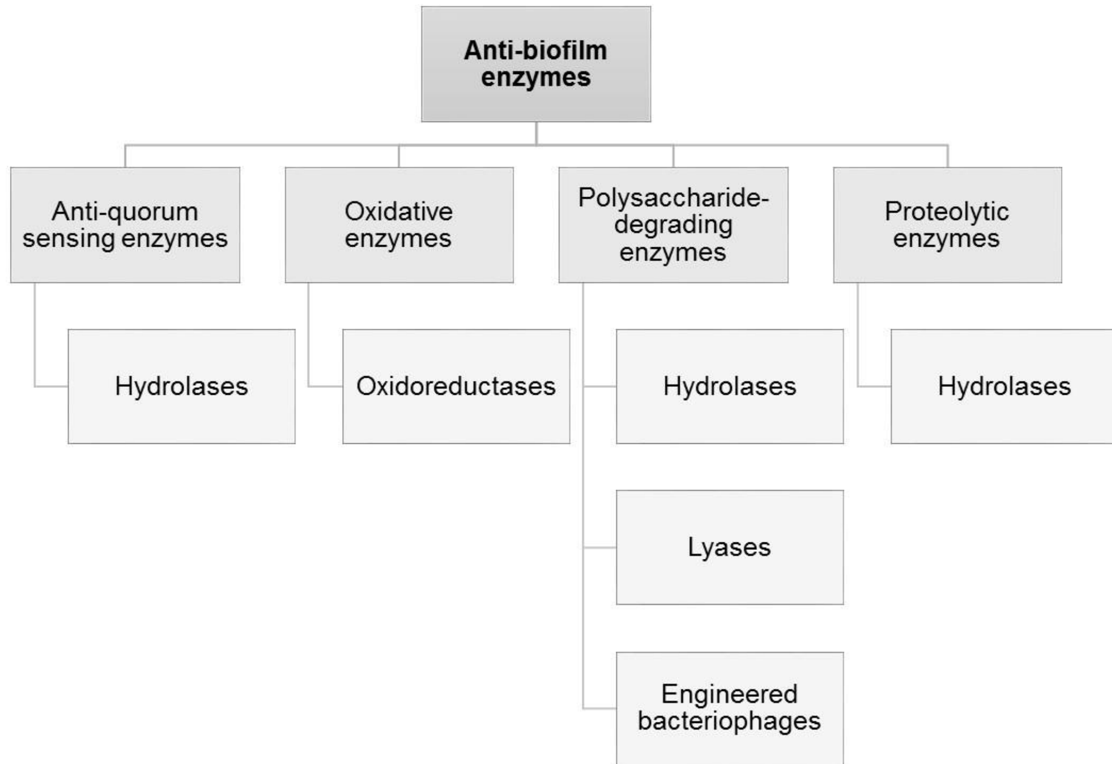
Yamasaki, M., Ogura, K., Hashimoto, W., Mikami, B., & Murata, K. (2005). A structural basis for depolymerization of alginate by polysaccharide lyase family-7. *Journal of Molecular Biology, 352*(1), 11-21.

Figure captions

Figure 1. Classification of enzymes relevant for biofilm control and detergent formulations (adapted from Thallinger et al. 2013).

Figure 2. Microscopy visualization of *E. coli* biofilm (5 days) on stainless steel (a), after application of Biorem A1 0.25% + Biorem 10 0.05% (Realco, Belgium) for 1 hour at 25 °C (b), and combination of the enzymatic treatment with 50 ppm sodium hypochlorite for 20 min at 25 °C (c). Magnification ×1000 and scale bar of 10 µm. Cells were stained with acridine orange 0.1 µg/mL (Sigma, Portugal). After 20 min of incubation in the dark, the slides were mounted with non-fluorescent immersion oil on glass microscope slides. The slides were examined using an epifluorescence microscope (LEICA DMLB2).

1



2

3 Figure 1

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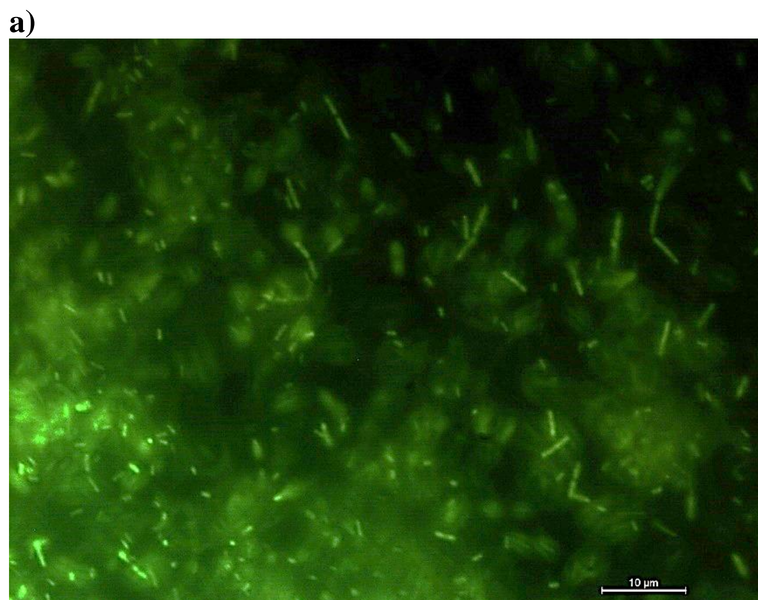
a)



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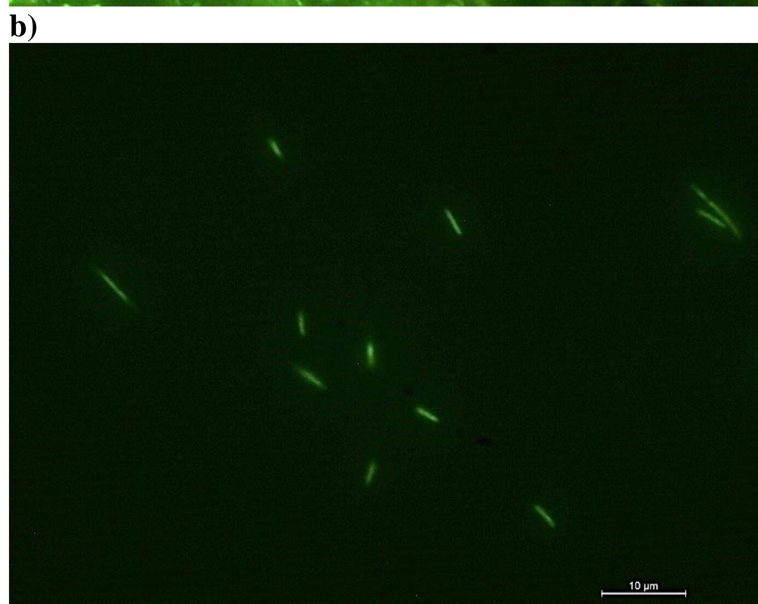

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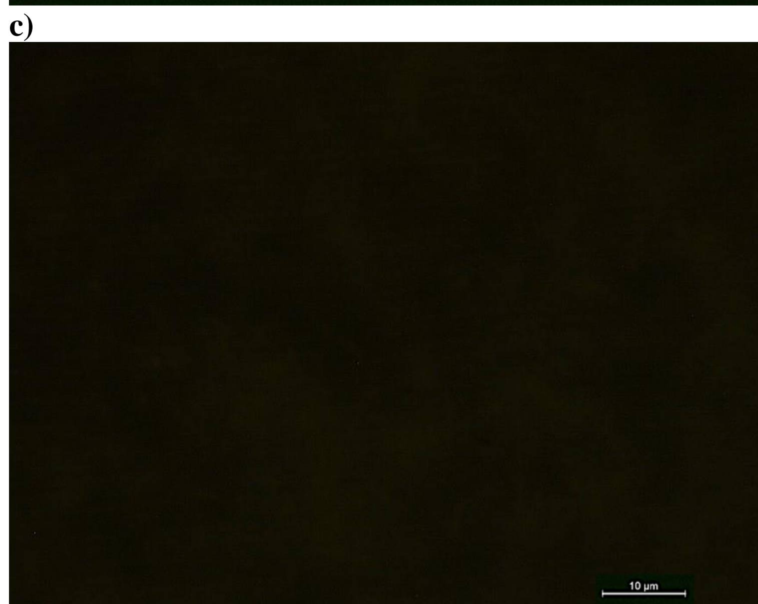

2 Figure 2

Table 2. Anti-biofilm applications of enzymes, their classification and targets

Enzyme	Enzyme class	Enzyme applied	Target biofilm producer	Surface material	Effect	Reference
Anti-QS enzymes	Hydrolase	Lactonase	*P. aeruginosa*	Polystyrene	69-77% biofilm removal	Kiran et al. (2011)
	Hydrolase	Acylase	Bacteria in a reverse osmosis membrane	Reverse osmosis membrane (material not specified)	9.0% biofilm removal	Kim et al. (2013)
	Hydrolase	Lactonase (expressed by an engineered T7 bacteriophage)	*P. aeruginosa* and *E. coli*	Polyvinyl chloride	Biofilm formation inhibition	Pei & Lamas-Samanamud (2014)
Oxidative enzymes	Hydrolase	DNase	*E. faecalis*	Polystyrene	Biofilm removal	Thomas et al. (2008)
	Hydrolase	DNase	*L. monocytogenes*	Polystyrene	50% biofilm removal	Nguyen & Burrows (2014)
Polysaccharide-degrading enzymes	Hydrolase	DispersinB	*S. epidermidis*	Glass	40% biofilm removal	Brindle et al. (2011)
	Hydrolase	α-amylase	*S. aureus*, *S. epidermidis*	Polystyrene	79% *S. aureus* biofilm removal; no biofilm removal for *S. epidermidis*	Craigen et al. (2011)
Proteolytic enzymes	Hydrolase	Pandion, Resinase, Spezyme and	*P. aeruginosa*	Polystyrene	4 log CFU/mL biofilm removal	Augustin et al. (2004)

		Paradigm used individually				
	Hydrolase	Bacteriophage enzyme	*E. coli* O157:H7	Stainless steel	Removal of 2.8 log CFU per stainless steell coupon	Sharma et al. (2005)
	Hydrolase	Bacteriophage enzyme	*E. coli*	Plastic pegs	99.997% removal	Lu & Collins (2007)
	Hydrolase	Pronase	*P. fluorescens*	Borosilicate glass	30% biofilm removal	Orgaz et al. (2007)
	Hydrolase	Savinase ®	*Pseudoalteromonas sp.*	Polystyrene	Complete biofilm removal	Leroy et al. (2008)
	Hydrolase	Savinase ®	*P. fluorescens*	Glass wool	80% biofilm removal	Molobela et al. (2010)
	Hydrolase	Endolysin (LysH5)	*S. aureus*	Polystyrene	1-3 log biofilm removal	Gutiérrez et al. (2014)
Anti QS + Proteolytic enzymes	Hydrolase	Acylase I + proteinase K	Bacteria in a reverse osmosis membrane	Reverse osmosis membrane (material not specified)	33.7% biofilm removal	Kim et al. (2013)
Oxidative + polysaccharide-degrading enzymes	Oxidoreductase + Hydrolase	Glucose oxidase + lactoperoxidase	*S. aureus*, *S. epidermidis*, *P. aeruginosa*, *P. fluorescens*	Stainless steel	1-2 log CFU/disc biofilm removal of *Staphylococcus*; 3 log CFU/disc biofilm removal of *Pseudomonas*	Johansen et al. (1997)
Proteolytic + polysaccharide-degrading enzymes	Hydrolase	Cellulase + Pronase	*P. fluorescens*	Borosilicate glass	94% of biofilm removal	Orgaz et al. (2007)

Proteolytic enzyme + shear stress	Hydrolase	Savinase® + shear stress	*P. aeruginosa*	Polyethylene	90% biofilm removal	Pechaud et al. (2012)
Proteolytic enzymes + ultrasounds	Hydrolase	Amyloglucosidas e + US	*E. coli*	Stainless steel	96% biofilm removal	Oulahal-Lagsir et al. (2003)
Polysaccharide -degrading enzymes + chemical treatment	Hydrolase	α-amylase + buffer with an anionic surfactant	*B. mycoides*	Stainless steel	2.98 log CFU/cm^2 biofilm removal	Lequette et al. (2010)
Polysaccharide -degrading enzymes + antibiotic	Lyase	Alginate lyase + gentamycin	*P. aeruginosa*	Cellulose fibbers	Complete biofilm removal	Alkawash et al. (2006)

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