Immobilized hydrolytic enzymes exhibit antibiofilm activity against *Escherichia coli* at sub-lethal concentrations

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Running headline: Antibiofilm activity of immobilized hydrolytic enzymes

Abstract

The effects of two commercially available immobilized enzymes (namely the glycosidase pectinase and the protease subtilisin A) at sub-lethal concentrations were investigated in terms of their influence on biofilm genesis, on the composition of the biofilm matrix, and their antibiotic synergy against *Escherichia coli* biofilm, used as a model system of bacterial biofilms. The best antibiofilm performance of solid-supported hydrolases was obtained at the surface concentration of 0.022 and 0.095 U/cm^2 with a reduction of 1.2 and 2.3 log CFU/biofilm for pectinase and subtilisin, respectively. At these enzyme surface concentrations, the biocatalysts affected the structural composition of the biofilm matrix, impacting biofilm thickness. Finally, the immobilized hydrolases enhanced biofilm sensitivity to a clinically relevant concentration of the antibiotic ampicillin. At the final antibiotic concentration of 0.1 mg/ml, a reduction of 2 and 3.5 $\log_{10}$ units in presence of 0.022 $U_{pectinase}$/cm^2 and 0.095 $U_{subtilisin}$/cm^2 was obtained respectively in comparison the antibiotic alone. Immobilized pectinase and subtilisin at sub-lethal concentrations demonstrated a great potential for antibiofilm applications.

Keywords: enzymes; *Escherichia coli* biofilm; antibiofilm performances; sub-lethal concentrations

Introduction

Any abiotic surface exposed to minimal conditions required for life is prone to microbial colonization leading to the development of surface-associated multicellular communities embedded in a self-produced polymeric matrix called biofilms. Biofilm microorganisms undergo processes of cell specialization, developing coordinated and efficient survival strategies [35].

Synthetic polymers do not escape from the problem that biofilms might cause. Well-known examples of unwanted biofilms on plastics include chronic infection of medical devices [53-54], microbial corrosion of pipelines and storage tanks [30, 56], biodeterioration of artistic materials [6, 14] and fouling in food processing equipment [5, 43, 48].

Despite the availability of control practices, the consequences of the biofilm mode of life are far-reaching. Microorganisms in biofilms exhibit increased tolerance toward antimicrobial agents, making some traditional biocide-based antibiofilm strategies ineffective [12, 18, 37]. The biofilm resistance to antimicrobials has profound consequences in our lifes, posing serious challenges to its eradication. Thus, to preserve surface functionality, guaranteeing suitable application lifetime, and to keep the growing human population in a healthy environment, new alternatives to replace or integrate the presently dominant antimicrobial regime are urgently required [50].

One strategy might be to destabilize biofilm organization and its physical integrity, disrupting its multicellular structure rather than affecting essential cellular functions that are crucial for microbial survival. In addition, if the multicellularity of the biofilm is compromised, the planktonic state might be forced, restoring the efficacy of antimicrobial agents. Potential strategies include enzymes that degrade the structural components of the biofilm matrix, compromising cohesiveness and destroying the backbone of the biofilm [22]. In addition, since biofilm matrix-degrading enzymes do not kill bacteria or inhibit their growth, the chances that resistance to these agents will evolve are reduced [20]. Finally, some enzymes are currently available at affordable prices and are therefore viable for industrial use, and since they are biodegradable and have a low toxicity, they might be attractive as environmentally friendly antibiofilm agents [8]. Starting from the assumption that polysaccharides and proteins are the major fractions of the matrix [13], hydrolytic enzymes such as glycosidases and proteases have been envisaged as interesting biofilm matrix-degrading agents [22].

Although the concept of using enzymes to counteract the formation of unwanted biofilms is not new, the scientific literature still lacks important information about the effects of immobilized biocatalysts at sub-lethal concentrations and their impacts on biofilm structural development. Until now, the attention has mainly focused on the antimicrobial activity of enzymes in solution, and such effects were investigated focusing the attention only on the initial surface attachment phase or on the biofilm dispersion phase [*inter alia* 24, 26-27, 29, 31, 58]. Few papers address the incorporation of enzymes into coatings yielding surfaces with antibiofilm spectrum [11, 36, 38, 44-45, 57]. However, even in these latter cases, the scientific community underestimated or neglected the impacts of immobilized enzymes at sub-lethal concentrations on biofilm structure and resistance to traditional antimicrobial agents.

In light of the previous considerations, the present work aimed to study the effects of two immobilized enzyme (namely the glycosidase pectinase and the protease subtilisin A) on i) biofilm genesis, ii) the structural composition of the biofilm matrix, iii) biofilm thickness and morphology, and iv) their antibiotic synergy against *Escherichia coli* biofilm, used as model system of bacterial biofilms. We demonstrated the antibiofilm performance of the two immobilized enzymes at sub-lethal concentrations, and their efficacy in destabilizing biofilm organization and its multicellular structure rather than affecting essential cellular functions that are crucial for microbial survival.

Materials and Methods

Materials. Pectinase from *Aspergillus niger* >1 U/mg solid (one unit (U) corresponds to the amount of enzyme which liberates 1 µmol galacturonic acid from polygalacturonic acid per minute at pH 4.0 and 50 °C), subtilisin from *Bacillus licheniformis* (subtilisin A) 8.6 U/mg solid (one U will hydrolyze casein to produce color equivalent to 1.0 µmole (181 µg) of tyrosine per min at pH 7.5 at 37 ° C (color by Folin-Ciocalteu reagent) and methoxypoly(ethylene glycol) (5

kDa) (PEG) were purchased from Sigma-Aldrich (Italy). Fifty % glutaraldehyde (GA) was obtained from Alfa-Aesar. Nitrocellulose membranes (0.45 μm) were purchased from Sigma Aldrich (N9763–5EA, Italy).

Enzyme immobilization. Pectinase and subtilisin were immobilized on nitrocellulose membranes (2.25 cm^2) by loading and fixing the biocatalysts by glutaraldehyde crosslinking in order to increase the stability and retention of the enzymes on the nitrocellulose membrane [3, 19, 23]. Seventy μl containing 45 μl of 0.02 mol/l potassium phosphate buffer, pH 7, with 0.025, 0.05, 0.1 or 0.2 mg enzyme and 25 μl polyethylene glycol (PEG) solution (4 g/l) were used. PEG acts as a stabilizing additive [42]. The final enzyme surface concentrations were 0.011, 0.022, 0.044, 0.088 U/cm^2 and 0.095, 0.189, 0.378, 0.757 U/cm^2 for pectinase and subtilisin, respectively. Aliquots of enzyme were taken from a 4 g/l stock solution. Next, 10 ml of glutaraldehyde (0.1% in the case of pectinase or 0.05% in the case subtilisin) were added, and just after, the final solution was loaded on the membrane. The membranes were allowed to dry overnight at 25 °C. Control membranes were prepared by the same procedures, without adding enzyme solution.

***Escherichia coli* strain and growth conditions**. The best characterized *Escherichia coli* strain K-12 (American Type Culture Collection ATCC 25404, wild type) was used throughout the study [39]. The microorganism was maintained at -80 °C in a suspension containing 40% glycerol and 4% peptone and it was routinely grown overnight in Luria-Bertani (LB, Sigma Aldrich, Italy) at 30°C.

Planktonic growth in presence of the enzymes both in solution and immobilized.

Planktonic growth of *E. coli* in LB medium supplemented with 2.86 mg/ml of hydrolases was carried out in 96-well microtiter plates as previously reported [49]. The experiments have been run with each enzyme separately. In addition, the immobilized enzymes at the maximum surface concentrations of 0.088 and 0.757 U/cm^2 for pectinase and subtilisin respectively (representing the concentration of a solution 2.86 mg/ml of hydrolases), were tested for their ability to affect the planktonic growth of *E. coli*. The membranes were immersed in 1 ml of a growth solution containing LB medium. The solution was inoculated with 50 μl (5% vol/vol) of an overnight culture of *E. coli*. Growth curves at 37°C were generated using the PowerWave XS2 microplate reader (Biotek). Growth was followed by measuring the optical density at 600 nm (OD_{600}) for over 24 h. OD-based growth kinetics were constructed by plotting the OD of suspensions minus the OD of the non-inoculated medium against incubation time. The polynomial Gompertz model [59] was used to fit the growth curves to calculate the maximum specific growth rate (μ_{max}) and lag time (λ), using GraphPad Prism software (version 5.0, San Diego, CA, USA). Three biological replicates of each treatment were performed.

Biofilm formation on enzyme-functionalized surfaces and biomass quantification. The effects of immobilized enzymes were studied using the agar-grown biofilm system (colony biofilms) representing static unsaturated biofilms [55]. In addition, this technique permitted us to directly investigate the effect of the immobilized enzymes on biofilm structural development and organization bypassing the effect on the adhesion phase [49]. Colony biofilms of *E. coli* were obtained as reported by Villa et al. [52] with few modifications. Briefly, 15 µl cell suspension containing 7.5 x 10^5 cells were used to inoculate untreated and treated nitrocellulose membranes resting on tryptic soy agar (TSA, Sigma Aldrich, Italy) culture medium. The plates were inverted and incubated at 30°C for 5 days, with the membrane-supported biofilm transferred to fresh culture medium every 24 h. Membranes were collected at the fifth day and transferred to 5 ml glass test tubes pre-filled with 5 ml sterile phosphate buffered saline (PBS, 10 mmol/l phosphate buffer, 0.3 mol/l NaCl pH 7.4 at 25 °C, Sigma-Aldrich, Italy). Biofilms were vortexed for 1 min to separate the cells from the membrane. In order to break apart clumps of cells, two cycles of 30 s at 20% power sonication (Branson 3510, Branson Ultrasonic Corporation, Dunburry, CT) followed by 30 s vortex mixing were applied. The resulting cell suspensions were serially diluted, plated on TSA, incubated 36 h at 30°C, and colony forming units (CFU) per membrane were enumerated using the drop-plate method [16].

Extraction and characterization of the extracellular polymeric matrix (EPS). Biofilm biomass was collected at the fifth day and resuspended in 2 ml 2% ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich, Italy). Biofilm cell suspensions were shaken at 300 rpm for 3 h at 4°C. After incubation, the samples were centrifuged for 20 min, 8000 x g at 4°C and the supernatant filtered through 0.2 µm polyethersulfone membranes (S623; Whatman, Inc., Florhan Park, NJ). Then, one half of the eluate was used for quantification of proteins and carbohydrates and cell lysis analysis, while the second half was used for extracellular DNA (eDNA) precipitation by the cetyltrimethylammonium bromide (CTAB)-DNA method as described by Corinaldesi et al. [9]. The method of Bradford [4] was applied for analyzing protein concentrations, whereas the optimized microplate phenol–sulfuric acid assay was applied for carbohydrate determination using glucose as the standard [33]. The results obtained were normalized by the weight of the wet biofilm biomass. Experiments were performed in triplicate.

Biofilm cryosectioning, staining and microscopic examination. Five days-old colony biofilms were covered carefully with a layer of Killik (Bio Optica, Italy) and placed on dry ice until completely frozen. Frozen samples were sectioned at -19°C using a Leitz 1720 digital cryostat (Leica, Italy). The 10-µm thick cryosections were mounted on glass slides treated with Vectabond (Vector laboratories, Italy), a non-protein tissue section adhesive. The lectin Concanavalin A-Texas Red conjugate (ConA, Invitrogen, Italy) was used to visualize the polysaccharide component of EPS, whereas the

amino-reactive dye Bodipy 630/650-X SE (Invitrogen, Italy) was used to visualize the protein in the EPS. Syto 9 green fluorescent nucleic acid stain (Invitrogen, Italy) was used to display biofilm cells. Biofilm sections were incubated with 200 µg/µl ConA and Bodipy and 5 mmol/l Sito-9 (Invitrogen) dye solution in PBS at room temperature in the dark for 30 min and then rinsed with PBS. Biofilm sections were visualized using a Leica TCSNT confocal laser scanning microscope with excitation at 488 nm, and emission ≥ 530 nm. Images were captured with a 10X/NA 0.45 dry lens objective and analyzed with the software Imaris (Bitplane Scientific Software, Zurich, Switzerland). The sections were also examined by fluorescence microscopy using a Leica DM 4000 B microscope at a magnification of 10X and biofilm thickness was measured as reported by Villa et al. [51].

Biofilm susceptibility assay. Powdered ampicillin was dissolved in sterile nanopure water, and the antibiotic solutions were added to the molten culture medium to create antibiotic-amended agar for biofilm experiments. The final antibiotic concentration used in biofilm assays was 0.1 mg/ml, a clinically relevant concentration. Antibiotic penetration of colony biofilms has been studied extensively suggesting the agent readily moves throughout the biofilm [60]. Five-day old biofilms were aseptically transferred to either antibiotic-containing agar or a control plate where they were incubated for an additional 24 h at room temperature. After this time, biofilm biomass was collected, physically disaggregated, serially diluted and plated on TSA as reported above. Antimicrobial efficacy was expressed as $\log_{10}$ microbial reduction. The $\log_{10}$ reduction was calculated relative to the cell count in the control samples without the antibiotic. All antimicrobial experiments were conducted in triplicate.

Statistical analysis. Analysis of variance (ANOVA) via a software run in MATLAB environment (Version 7.0, The MathWorks Inc, Natick, USA) was applied to statistically evaluate any significant differences among the samples. Tukey's honestly significant different test (HSD) was used for pairwise comparison to determine the significance of the data. Statistically significant results were depicted by p-values < 0.05.

Results and Discussion

Hydrolases are known to have antibiofilm properties against both gram-positive and gram-negative bacteria. They successfully counteract both biofilms from the paper industry [31] and invasion ability and biofilm formation in *Listeria monocytogenes* [29]. They also show a wide antifouling activity against different bacterial strains isolated from food-processing lines [25] and inhibit the extent of co-aggregation of *Actinomyces naeslundii*, *Streptococcus oralis*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* [24]. However, in these scientific works, the antibiofilm

165 performances of the enzymes were investigated in solution, underestimating the effectiveness of immobilized enzymes
166 at sub-lethal concentrations to resist biofilm formation over a working timescale.
167 Before evaluating the antibiofilm performance of immobilized pectinase and subtilisin, we studied their impact on *E.*
168 *coli* planktonic growth (Table 1). In this work, the hydrolases, both in solution and immobilized, did not affect bacterial
169 growth at the concentrations tested, showing their potential as biocide-free antibiofilm strategy.
170 The results of the antibiofilm activity of immobilized enzymes are presented in Fig. 1. The best antibiofilm performance
171 of immobilized hydrolases was obtained at the surface concentration of 0.022 and 0.095 U/cm^2 with a reduction of 1.2
172 and 2.3 log CFU/biofilm for pectinase and subtilisin, respectively. In addition, the results suggested that subtilisin is
173 more effective in hindering biofilm formation of *E. coli* than pectinase. Noteworthy was the observation that the best
174 antibiofilm performances of both the immobilized enzymes were obtained at a specific threshold level, which does not
175 correspond to the maximum enzyme surface concentration tested. Overall, these results demonstrated that hydrolases
176 could either reduce biofilm biomass effectively, or conversely promote biofilm growth, depending on the enzymatic
177 concentrations tested [26]. The non-linear response patterns followed a parabola-like shape profile, resembling a
178 hormetic property, a situation in which the response to an environmental stressor varies with the level of exposure [50].
179 This adaptive response not only enhances survival by providing resistance to environmental stresses, but it also helps
180 regulate the allocation of resources in a manner that ensures stability and fitness of cells [50]. As the biofilm lifestyle is
181 considered an adaptive response of microorganisms to cope with a harsh environment, likely high sub-lethal
182 concentrations of enzymes might induce a direct or indirect stress, stimulating biofilm formation.
183 The hormesis phenomenon is not new in the biofilm world. Villa and colleagues [51] observed that the best antibiofilm
184 performance of sub-lethal concentrations of the phenolic compound zosteric acid against *Candida albicans* biofilm was
185 obtained at the specific concentration of 10 mg/l. As the concentration fell below or above that threshold level, an
186 increase in biofilm biomass was observed. The biphasic profile is also induced by the biofilm mediators homoserine
187 lactones, which act in a concentration-dependent manner, where upper and lower threshold concentrations trigger the
188 formation of a biofilm [40].
189 The recent demonstration that antibiotics exert the phenomenon of hormesis provides a further explanation for the dual
190 activities of microbially derived natural products like enzymes. Migliore and colleagues [34] showed the ability of sub-
191 inhibitory concentrations of tetracycline to induce a hormetic response in the model organism *E. coli* MG1655. The
192 results demonstrated that low concentrations of tetracycline led to an increase in the biomass, and the dose-response
193 curve describing this numerical increase is an inverted-U-shaped curve. Such dose-response dependence has been
194 demonstrated by several published studies, reporting that at high concentrations, antibiotics eradicate bacteria, while at
195 low concentrations biofilm formation is induced [17, 28, 41]. These findings confirm that hormesis is common to many

196 living systems, including bacteria, underlying the need of an in-depth knowledge of both the effects and the possible
197 consequences of exposure to different doses of bioactive molecules, including enzymes.
198 The EPS matrix is the main component of biofilms and plays several roles in their life that can be listed as constructive,
199 informative, sorptive, (redox)-active, surface active, and nutritive functions [13]. The matrix is involved in the adhesion
200 of biofilms to surfaces, mediating the mechanical stability of biofilms and determining biofilm architecture [47]. The
201 effects of immobilized enzymes on EPS composition were investigated by comparing the content of proteins, total
202 polysaccharides, and extracellular DNA of EPS (Fig. 2). The investigation showed that mainly proteins and
203 polysaccharides compose the biofilm matrix of *E. coli* biofilm, as no detectable amount of extracellular DNA was
204 measured. With the immobilized pectinase at the surface concentration of 0.022 U/cm^2, the extracellular protein and
205 polysaccharide contents were reduced by 91.8% and 85.7%, respectively (Fig. 2a). A significant reduction in protein (-
206 61.4%) and polysaccharide concentrations (-76.1%) was also observed with the subtilisin at the surface concentration of
207 0.095 U/cm^2 as compared with the respective control (Fig. 2b).
208 The results obtained by the biochemical analysis of the matrix were further confirmed by microscopic examination of
209 biofilm cryosections (Fig. 3). Images captured from frozen sections showed that biofilms grown on the immobilized
210 enzymes retained similar morphological patterns as those grown on the control. However, a reduction in the fluorescent
211 signals corresponding to the protein and polysaccharide contents was observed in the treated samples. *E. coli* biofilms
212 exposed to immobilized enzymes were significantly thinner (biofilm thickness$_{(\text{protease 0,022 U/cm2})}$: 239.5 ± 24.1 μm; biofilm
213 thickness$_{(\text{subtilisin 0,095 U/cm2})}$: 225.7 ± 25.2 μm) than the biofilm grown on the control (biofilm thickness 334 ± 28.2 μm),
214 corroborating the ability of the immobilized biocatalysts to reduce biofilm biomass.
215 Our findings suggested that the mechanisms by which the immobilized enzymes might exert their antibiofilm effects
216 include the degradation of the matrix, thereby weakening the biofilm structure. Leroy and colleagues [27] reported that
217 free subtilisin was more effective in inhibiting adhesion than in enabling biofilm detachment of the marine bacteria
218 *Pseudoalteromonas* sp. D41, suggesting its ineffectiveness on structural composition of the biofilm matrix. In contrast,
219 Hangler *et al.* [15] observed that the serine protease Esperase HPF (subtilisin) affected both the attachment and the
220 detachment of a multispecies biofilm, suggesting that, in this case, the enzyme effectively degraded both protein-based
221 adhesives and proteins contained in the matrix. Recent work also showed that differences in the chemical composition
222 of the EPS are reflected in the vulnerability of biofilms to enzymatic treatments [2, 7, 25].
223 It is widely recognized that the susceptibility of *E. coli* biofilm to many conventional antimicrobial agents is reduced
224 compared to the susceptibility of planktonic cells. Therefore, the sensitivity of biofilms grown in the presence of the
225 immobilized hydrolases was examined to determine whether the same recalcitrance occurred. The graphs reported in
226 Fig. 4a-b showed a biofilm reduction of 2 and 3.5 $\log_{10}$ units in presence of 0.022 U/cm^2 pectinase and 0.095 U/cm^2

subtilisin, respectively, when exposed to clinically relevant concentration of ampicillin. As expected, the heavily perturbed matrix of biofilms grown on immobilized enzymes increased the activity of the antibiotic ampicillin. Darouiche and colleagues [10] reported that the combination of the antiseptic triclosan and the enzymatic product Dispersin B in solution showed synergistic antimicrobial and antibiofilm activity against *Staphylococcus aureus, S. epidermidis* and *E. coli*. Co-administration of alginate lyase (20 U/ml) with gentamicin (64 μg/ml) increased the killing of biofilms of mucoid *P. aeruginosa* growing in conditions similar to those found in the respiratory tract [1]. Tetz *et al.* [46] reported a strong negative impact of deoxyribonuclease I (DNase I) on the structures of biofilms formed by *Acinetobacter baumannii*, *Haemophilus influenzae*, *K. pneumoniae, E. coli, P. aeruginosa, S. aureus,* and *Streptococcus pyogenes*. Azithromycin, rifampin, levofloxacin, ampicillin, and cefotaxime were more effective in the presence of DNase I (5 μg/ml). Furthermore, the antibiofilm activity of deoxyribonuclease I (130 μg/ml) in combination with selected antibiotics toward *C. albicans* biofilms was estimated [32]. A reduction of viable counts by 0.5 $\log_{10}$ units was observed for biofilm-growing *C. albicans* incubated with DNase I. Treating *C. albicans* with amphotericin B alone (1 μg/ml) resulted in a 1 $\log_{10}$ unit reduction in cell viability, which increased to 3.5 $\log_{10}$ units in combination with DNase I. Cell viability was reduced by 5 $\log_{10}$ units at higher concentrations of amphotericin B (>2 μg /ml) and DNase I [32]. Kiran et al. [21] identified lactonase as a potential antibiofilm agent, as 0.3 U/ml of the enzyme disrupted the biofilm structure and led to increased ciprofloxacin and gentamycin penetration and antimicrobial activity. However, all the enzymes were tested in solution and no information was available about their lethal concentrations. Thus, the present work represents an important step forward in the development of antibiofilm materials, showing the synergistic effects of immobilized hydrolytic enzymes (at sub-lethal concentrations) and antibiotics on *E. coli*.

Therefore, solid-supported hydrolytic enzymes considered in this study might hold great potential for antibiofilm applications in both the medical and industrial domains. Future works will aim at evaluating the antibiofilm performance of the two enzymes together once immobilized onto a polymeric surface at sub-lethal concentrations.

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253 **References**

254 1. Alkawash MA, Soothill JS, Schiller NL (2006) Alginate lyase enhances antibiotic killing of mucoid
255 *Pseudomonas aeruginosa* in biofilms. APMIS 114:131-138. doi:10.1128/AAC.01789-12
256 2. Augustin M, Ali-Vehmas T, Atroshi F (2004) Assessment of enzymatic cleaning agents and disinfectants
257 against bacterial biofilms. J Pharm Pharm Sci 7:55-64.
258 3. Barbosa O, Ortiz C, Berenguer-Murcia A, Torres R, Rodrigues RC, Fernandez-Lafuente R (2014)
259 Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization.
260 RSC Adv 4:1583-1600. doi: 10.1039/C3RA45991H
261 4. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein
262 utilizing the principle of protein-dye binding. Anal Biochem 72:248-254. doi:10.1016/0003-2697(76)90527-3
263 5. Cappitelli F, Polo A, Villa F (2014) Biofilm formation in food processing environments is still poorly
264 understood and controlled. Food Eng Rev 6:29-42. doi:10.1007/s12393-014-9077-8
265 6. Cappitelli F, Zanardini E, Sorlini C (2004) The biodeterioration of synthetic resins used in conservation.
266 Macromol Biosci **4**:399-406. doi:10.1002/mabi.200300055
267 7. Chaignon P, Sadovskaya I, Ragunah Ch, Ramasubbu N, Kaplan JB, Jabbouri S (2007) Susceptibility of
268 staphylococcal biofilms to enzymatic treatments depends on their chemical composition. Appl Microbiol
269 Biotechnol 75:125-132. doi:10.1007/s00253-006-0790-y
270 8. Cordeiro AL, Hippius C, Werner C (2011) Immobilized enzymes affect biofilm formation. Biotechnol Lett
271 33:1897-1904. doi:10.1007/s10529-011-0643-3
272 9. Corinaldesi C, Danovaro R, Dell'Anno A (2005) Simultaneous recovery of extracellular and intracellular DNA
273 suitable for molecular studies from marine sediments. Appl Environ Microbiol 71:46-50.
274 doi:10.1128/AEM.71.1.46-50.2005
275 10. Darouiche RO, Mansouri MD, Gawande PV, Madhyastha S (2009) Antimicrobial and antibiofilm efficacy of
276 triclosan and DispersinB combination. J Antimicrob Chemother 64:88-93. doi:10.1093/jac/dkp158
277 11. Elchinger PH, Delattre C, Faure S, Roy O, Badel S, Bernardi T, Taillefumier C, Michaud P (2015)
278 Immobilization of proteases on chitosan for the development of films with anti-biofilm properties. Int J Biol
279 Macromol 72:1063-1068. doi: 10.1016/j.ijbiomac.2014.09.061
280 12. Flemming HC (2011) Microbial biofouling: unsolved problems, insufficient approaches, and possible
281 solutions. In Flemming HC et al. (eds.), Biofilm Highlights, Springer Series on Biofilms 5, Springer-Verlag
282 Berlin Heidelberg

13. Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623-633. doi:10.1038/nrmicro2415
14. Giacomucci L, Toja F, Sanmartín P, Toniolo L, Prieto B, Villa F, Cappitelli F (2012) Degradation of nitrocellulose-based paint by *Desulfovibrio desulfuricans* ATCC 13541. Biodegradation 23.705-716. doi:10.1007/s10532-012-9546-9
15. Hangler M, Burmølle M, Schneider I, Allermann K, Jensen B (2009) The serine protease Esperase HPF inhibits the formation of multispecies biofilm. Biofouling 25:667-674. doi:10.1080/08927010903096008.
16. Herigstad B, Hamilton M, Heersink J (2001) How to optimize the drop plate method for enumerating bacteria. J Microbiol Methods 44:121-129. doi:10.1016/S0167-7012(00)00241-4
17. Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. Nature 436:1171-1175. doi:10.1038/nature03912
18. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010) Antibiotic resistance of bacterial biofilms. *Int J* Antimicrob Agents 35:322-332. doi:10.1016/j.ijantimicag.2009.12.011
19. Ikegaki N, Kennett RH (1989) Glutaraldehyde fixation of the primary antibody-antigen complex on nitrocellulose paper increases the overall sensitivity of immunoblot assay. J Immunol Methods 124:205-210. doi:10.1016/0022-1759(89)90354-2
20. Kaplan JB (2009) Therapeutic potential of biofilm-dispersing enzymes. Int J Artif Organs 32.545-554.
21. Kiran S, Sharma P, Harjai K, Capalash N (2011) Enzymatic quorum quenching increases antibiotic susceptibility of multidrug resistant *Pseudomonas aeruginosa*. Iran J Microbiol 3:1-12.
22. Kristensen JB, Meyer RL, Laursen BS, Shipovskov S, Besenbacher F, Poulsen CH (2008) Antifouling enzymes and the biochemistry of marine settlement. Biotechnol Adv 26:471-481. doi:10.1016/j.biotechadv.2008.05.005
23. Kumar A, Kanwar SS (2012) Catalytic potential of a nitrocellulose membrane-immobilized lipase in aqueous and organic media. J App Polym Sci 124:E37-E44. doi:10.1002/app.35434
24. Ledder RG, Madhwani T, Sreenivasan PK, De Vizio W, McBain AJ (2009) An in vitro evaluation of hydrolytic enzymes as dental plaque control agents. J Med Microbiol 58:482-491. doi:10.1099/jmm.0.006601-0
25. Lequette Y, Boels G, Clarisse M, Faille C (2010) Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. Biofouling 26:421-431. doi:10.1080/08927011003699535
26. Leroy C, Delbarre C, Ghillebaert F, Compere C, Combes D (2008) Effects of commercial enzymes on the adhesion of a marine biofilm-forming bacterium. Biofouling 24:11-22. doi:10.1080/08927010701784912

27. Leroy C, Delbarre C, Ghillebaert F, Compere C, Combes D (2008b) Influence of subtilisin on the adhesion of a marine bacterium which produces mainly proteins as extracellular polymers. J Appl Microbiol 105:791-799. doi:10.1111/j.1365-2672.2008.03837.x

28. Linares JF, Gustafsson I, Baquero F, Martinez JL (2006) Antibiotics as intermicrobial signaling agents instead of weapons. Proc Natl Acad Sci USA 103:19484–19489. doi:10.1073/pnas.0608949103

29. Longhi C, Scoarughi GL, Poggiali F, Cellini A, Carpentieri A, Seganti L, Pucci P, Amoresano A, Cocconcelli PS, Artini M, Costerton JW Selan L (2008) Protease treatment affects both invasion ability and biofilm formation in *Listeria monocytogenes*. Microb Pathog 45:45-52. doi:10.1016/j.micpath.2008.01.007

30. Lopes FA, Morin P, Oliveira R, Melo LF (2006) Interaction of *Desulfovibrio desulfuricans* biofilms with stainless steel surface and its impact on bacterial metabolism. J Appl Microbiol 101:1087-1095. doi:10.1111/j.1365-2672.2006.03001.x

31. Marcato-Romain CE, Pechaud Y, Paul E, Girbal-Neuhauser E, Dossat-Létisse V (2012) Removal of microbial multi-species biofilms from the paper industry by enzymatic treatments. Biofouling 28:305-314. doi:10.1080/08927014.2012.673122

32. Martins M, Henriques M, Lopez-Ribot JL, Oliveira R (2012) Addition of DNase improves the in vitro activity of antifungal drugs against *Candida albicans* biofilms. Mycoses 55:80-85. doi:10.1111/j.1439-0507.2011.02047.x

33. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S, Lee YC (2005) Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem 339:69-72. doi:10.1016/j.ab.2004.12.001

34. Migliore L, Rotini A, Thaller MC (2013) Low doses of tetracycline trigger the *E. coli* growth: a case of hormetic response. Dose Response 11:550-557. doi:10.2203/dose-response.13-002.Migliore. eCollection 2013

35. Pace JL, Rupp ME, Finch RG (2006) Biofilms, Infection, and Antimicrobial Therapy. FL: CRC Press, Taylor & Francis Group.

36. Pavlukhina SV, Kaplan JB, Xu L, Chang W, Yu X, Madhyastha S, Yakandawala N, Mentbayeva A, Khan B, Sukhishvili SA (2012) Noneluting enzymatic antibiofilm coatings. ACS Appl Mater Interfaces 4:4708-4716. doi:10.1021/am3010847

37. Polo A, Diamanti MV, Bjarnsholt T, Høiby N, Villa F, Pedeferri MP, Cappitelli F (2011) Effects of photoactivated titanium dioxide nanopowders and coating on planktonic and biofilm growth of *Pseudomonas aeruginosa*. Photochem Photobiol 87:1387-1394. doi:10.1111/j.1751-1097.2011.00972.x

38. Regina VR, Søhoel H, Lokanathan AR, Bischoff C, Kingshott P, Revsbech NP, Meyer RL (2012) Entrapment of subtilisin in ceramic sol-gel coating for antifouling applications. ACS Appl Mater Interfaces 4:5915-5921. doi:10.1021/am301554m

39. Ren D, Zuo R, González Barrios AF, Bedzyk LA, Eldridge GR, Pasmore ME, Wood TK (2005) Differential gene expression for investigation of *Escherichia coli* biofilm inhibition by plant extract ursolic acid. Appl Environ Microbiol 71:4022-4034. doi:10.1128/AEM.71.7.4022-4034.2005

40. Rickard AH, Palmer RJJr, Blehert DS, Campagna SR, Semmelhack MF, Egland PG, Bassler BL, Kolenbrander PE (2006) Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. Mol Microbiol 60:1446-1456. doi:10.1111/j.1365-2958.2006.05202.x

41. Salta M, Wharton JA, Dennington SP, Stoodley P, Stokes KR (2013) Antibiofilm performance of three natural products against initial bacterial attachment. Int J Mol Sci 14:21757-21780. doi:10.3390/ijms141121757

42. Secundo F, Barletta GL, Parini G, Roda G (2012) Effects of stabilizing additives on the activity of alpha-chymotrypsin in organic solvent. J Mol Catal B: Enzym 84:128-131. doi:10.1016/j.molcatb.2012.04.008

43. Srey S, Jahid IK, Ha SD (2013) Biofilm formation in food industries: A food safety concern. Food Control 31:572-585. doi:10.1016/j.foodcont.2012.12.001

44. Swartjes JJTM, Das T, Sharifi S, Subbiahdoss G, Sharma PK, Krom BP, Busscher HJ, van der Mei HC (2013) A functional DNase I coating to prevent adhesion of bacteria and the formation of biofilm. Adv Funct Mater 23:2843–2849. doi:10.1002/adfm.201202927

45. Tasso M, Pettitt ME, Cordeiro AL, Callow ME, Callow JA, Werner C (2009) Antifouling potential of Subtilisin A immobilized onto maleic anhydride copolymer thin films. Biofouling 25:505-516. doi:10.1080/08927010902930363

46. Tetz GV, Artemenko NK, Tetz VV (2009) Effect of DNase and antibiotics on biofilm characteristics. Antimicrob Agents Chemother 53:1204-1209. doi:10.1128/AAC.00471-08

47. Tielen P, Rosenau F, Wilhelm S, Jaeger KE, Flemming HC, Wingender J (2010) Extracellular enzymes affect biofilm formation of mucoid *Pseudomonas aeruginosa*. Microbiology 156:2239-2252. doi:10.1099/mic.0.037036-0

48. Vázquez-Sánchez D, Habimana O, Holck A (2013) Impact of food-related environmental factors on the adherence and biofilm formation of natural *Staphylococcus aureus* isolates. Curr Microbiol 66:110-121. doi:10.1007/s00284-012-0247-8

49. Villa F, Albanese D, Giussani B, Stewart PS, Daffonchio D, Cappitelli F (2010) Hindering biofilm formation with zosteric acid. Biofouling 26:739-752. doi:10.1080/08927014.2010.511197

50. Villa F, Cappitelli F (2013) Plant-derived bioactive compounds at sub-lethal concentrations: towards smart biocide-free antibiofilm strategies. Phytochem Rev 12:245-254. doi:10.1007/s11101-013-9286-4
51. Villa F, Pitts B, Stewart PS, Giussani B, Roncoroni S, Albanese D, Giordano C, Tunesi M, Cappitelli F (2011) Efficacy of zosteric acid sodium salt on the yeast biofilm model *Candida albicans*. Microb Ecol 62:584-598.
52. Villa F, Remelli W, Forlani F, Gambino M, Landini P, Cappitelli F (2012) Effects of chronic sub-lethal oxidative stress on biofilm formation by *Azotobacter vinelandii*. Biofouling 28:823-833. doi:10.1080/08927014.2012.715285
53. Villa F, Villa S, Gelain A, Cappitelli F (2013) Sub-lethal activity of small molecules from natural sources and their synthetic derivatives against biofilm forming nosocomial pathogens. Curr Top Med Chem 13:3184-3204. doi:10.2174/15680266113136660225
54. Wang X, Lünsdorf H, Ehrén I, Brauner A, Römling U (2010) Characteristics of biofilms from urinary tract catheters and presence of biofilm-related components in *Escherichia coli*. Curr Microbiol 60:446-53. doi: 10.1007/s00284-009-9563-z
55. Wingender J, Strathmann M, Rode A, Leis A, Flemming HC (2001). Isolation and biochemical characterization of extracellular polymeric substances from *Pseudomonas aeruginosa*. Methods Enzymol 336:302–314. doi:10.1016/S0076-6879(01)36597-7
56. Yu J, Kim D, Lee T (2010) Microbial diversity in biofilms on water distribution pipes of different materials. Water Sci Technol 61:163-171. doi:10.2166/wst.2010.813
57. Yuan S, Yin J, Jiang W, Liang B, Pehkonen SO, Choong C (2013). Enhancing antibacterial activity of surface-grafted chitosan with immobilized lysozyme on bioinspired stainless steel substrates. Colloids Surf B Biointerfaces 106:11-21. doi: 10.1016/j.colsurfb.2012.12.048
58. Zanaroli G, Negroni A, Calisti C, Ruzzi M, Fava F (2011) Selection of commercial hydrolytic enzymes with potential antifouling activity in marine environments. Enzyme Microb Technol 49:574-579. doi:10.1016/j.enzmictec.2011.05.008
59. Zuroff TR, Bernstein H, Lloyd-Randolfi J, Jimenez-Taracido L, Stewart PS, Carlson RP (2010) Robustness analysis of culturing perturbations on *Escherichia coli* colony biofilm beta-lactam and aminoglycoside antibiotic tolerance. BMC Microbiol 10:185. doi:10.1186/1471-2180-10-185
60. Zwietering MH, Jongenburger I, Rombouts FM van't Riet K (1990) Modeling of the bacterial growth curve. Appl Environ Microbiol 56:1875–1881.

405 **Tables and Figures**

406

407 **Table 1:** The table summarizes the growth parameters lag time (λ) and maximum growth rate (μ max) and the
408 Goodness of Fit (R^2) obtained by the Gombertz models. Different superscript letters indicate significant differences
409 (Tukey's HSD, $p<0.05$) between the means of three independent replicates.

410

411 **Figure 1:** Effects of immobilized enzymes on biofilm growth. Data represent the mean ± standard deviation of three
412 independent measurements. The graph provides the p-values obtained by ANOVA analysis. According to post-hoc
413 analysis (Tukey's HSD, $p<0.05$), means sharing the same letter are not significantly different from each other.

414

415 **Figures 2:** Effects of immobilized enzymes on EPS composition. Data represent the mean ± standard deviation of three
416 independent measurements. The graph provides the p-values obtained by ANOVA analysis. According to post-hoc
417 analysis (Tukey's HSD, $p<0.05$), means sharing the same letter are not significantly different from each other.

418

419 **Figures 3:** Cryosectioning images of *E. coli* biofilms grown without and with the immobilized enzymes. Live cells
420 were stained in green with Syto9, whereas the polysaccharide (a-b) or the protein (c-d) components of the biofilm
421 matrix were stained in red. Scale bars represent 150 μm.

422

423 **Figure 4**: Effects of immobilized enzymes on antibiotic resistance of *E. coli* biofilm. The graphs report the value of
424 $\log_{10}$ unit reductions. Data represent the mean ± standard deviation of three independent measurements. The graph
425 provides the *p*-values obtained by ANOVA analysis. According to post-hoc analysis (Tukey's HSD, $p<0.05$), means
426 sharing the same letter are not significantly different from each other.