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/cm² 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}/cm² and 0.095 U_{subtilisin}/cm² 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 cm²) 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/cm² and 0.095, 0.189, 0.378, 0.757 U/cm² 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/cm² 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 (OD₆₀₀) 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 (µₘₐₓ) 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., Florham 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 Concanaavalin 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
performances of the enzymes were investigated in solution, underestimating the effectiveness of immobilized enzymes at sub-lethal concentrations to resist biofilm formation over a working timescale.

Before evaluating the antibiofilm performance of immobilized pectinase and subtilisin, we studied their impact on *E. coli* planktonic growth (Table 1). In this work, the hydrolases, both in solution and immobilized, did not affect bacterial growth at the concentrations tested, showing their potential as biocide-free antibiofilm strategy.

The results of the antibiofilm activity of immobilized enzymes are presented in Fig. 1. The best antibiofilm performance of immobilized hydrolases was obtained at the surface concentration of 0.022 and 0.095 U/cm² with a reduction of 1.2 and 2.3 log CFU/biofilm for pectinase and subtilisin, respectively. In addition, the results suggested that subtilisin is more effective in hindering biofilm formation of *E. coli* than pectinase. Noteworthy was the observation that the best antibiofilm performances of both the immobilized enzymes were obtained at a specific threshold level, which does not correspond to the maximum enzyme surface concentration tested. Overall, these results demonstrated that hydrolases could either reduce biofilm biomass effectively, or conversely promote biofilm growth, depending on the enzymatic concentrations tested [26]. The non-linear response patterns followed a parabola-like shape profile, resembling a hormetic property, a situation in which the response to an environmental stressor varies with the level of exposure [50].

This adaptive response not only enhances survival by providing resistance to environmental stresses, but it also helps regulate the allocation of resources in a manner that ensures stability and fitness of cells [50]. As the biofilm lifestyle is considered an adaptive response of microorganisms to cope with a harsh environment, likely high sub-lethal concentrations of enzymes might induce a direct or indirect stress, stimulating biofilm formation.

The hormesis phenomenon is not new in the biofilm world. Villa and colleagues [51] observed that the best antibiofilm performance of sub-lethal concentrations of the phenolic compound zosteric acid against *Candida albicans* biofilm was obtained at the specific concentration of 10 mg/l. As the concentration fell below or above that threshold level, an increase in biofilm biomass was observed. The biphasic profile is also induced by the biofilm mediators homoserine lactones, which act in a concentration-dependent manner, where upper and lower threshold concentrations trigger the formation of a biofilm [40].

The recent demonstration that antibiotics exert the phenomenon of hormesis provides a further explanation for the dual activities of microbially derived natural products like enzymes. Migliore and colleagues [34] showed the ability of sub-inhibitory concentrations of tetracycline to induce a hormetic response in the model organism *E. coli* MG1655. The results demonstrated that low concentrations of tetracycline led to an increase in the biomass, and the dose-response curve describing this numerical increase is an inverted-U-shaped curve. Such dose-response dependence has been demonstrated by several published studies, reporting that at high concentrations, antibiotics eradicate bacteria, while at low concentrations biofilm formation is induced [17, 28, 41]. These findings confirm that hormesis is common to many
living systems, including bacteria, underlying the need of an in-depth knowledge of both the effects and the possible consequences of exposure to different doses of bioactive molecules, including enzymes.

The EPS matrix is the main component of biofilms and plays several roles in their life that can be listed as constructive, informative, sorptive, (redox)-active, surface active, and nutritive functions [13]. The matrix is involved in the adhesion of biofilms to surfaces, mediating the mechanical stability of biofilms and determining biofilm architecture [47]. The effects of immobilized enzymes on EPS composition were investigated by comparing the content of proteins, total polysaccharides, and extracellular DNA of EPS (Fig. 2). The investigation showed that mainly proteins and polysaccharides compose the biofilm matrix of *E. coli* biofilm, as no detectable amount of extracellular DNA was measured. With the immobilized pectinase at the surface concentration of 0.022 U/cm², the extracellular protein and polysaccharide contents were reduced by 91.8% and 85.7%, respectively (Fig. 2a). A significant reduction in protein (-61.4%) and polysaccharide concentrations (-76.1%) was also observed with the subtilisin at the surface concentration of 0.095 U/cm² as compared with the respective control (Fig. 2b).

The results obtained by the biochemical analysis of the matrix were further confirmed by microscopic examination of biofilm cryosections (Fig. 3). Images captured from frozen sections showed that biofilms grown on the immobilized enzymes retained similar morphological patterns as those grown on the control. However, a reduction in the fluorescent signals corresponding to the protein and polysaccharide contents was observed in the treated samples. *E. coli* biofilms exposed to immobilized enzymes were significantly thinner (biofilm thickness* (protease 0.022 U/cm²): 239.5 ± 24.1 µm; biofilm thickness* (subtilisin 0.095 U/cm²): 225.7 ± 25.2 µm) than the biofilm grown on the control (biofilm thickness 334 ± 28.2 µm), corroborating the ability of the immobilized biocatalysts to reduce biofilm biomass.

Our findings suggested that the mechanisms by which the immobilized enzymes might exert their antibiofilm effects include the degradation of the matrix, thereby weakening the biofilm structure. Leroy and colleagues [27] reported that free subtilisin was more effective in inhibiting adhesion than in enabling biofilm detachment of the marine bacteria *Pseudoalteromonas* sp. D41, suggesting its ineffectiveness on structural composition of the biofilm matrix. In contrast, Hangler *et al.* [15] observed that the serine protease Esperase HPF (subtilisin) affected both the attachment and the detachment of a multispecies biofilm, suggesting that, in this case, the enzyme effectively degraded both protein-based adhesives and proteins contained in the matrix. Recent work also showed that differences in the chemical composition of the EPS are reflected in the vulnerability of biofilms to enzymatic treatments [2, 7, 25].

It is widely recognized that the susceptibility of *E. coli* biofilm to many conventional antimicrobial agents is reduced compared to the susceptibility of planktonic cells. Therefore, the sensitivity of biofilms grown in the presence of the immobilized hydrolases was examined to determine whether the same recalcitrance occurred. The graphs reported in Fig. 4a-b showed a biofilm reduction of 2 and 3.5 log$_{10}$ units in presence of 0.022 U/cm² pectinase and 0.095 U/cm² pectinase.
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 biofilm 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 log10 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 log10 unit reduction in cell viability, which increased to 3.5 log10 units in combination with DNase I. Cell viability was reduced by 5 log10 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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Tables and Figures

Table 1: The table summarizes the growth parameters lag time ($\lambda$) and maximum growth rate ($\mu_{\text{max}}$) and the Goodness of Fit ($R^2$) obtained by the Gomertz models. Different superscript letters indicate significant differences (Tukey's HSD, $p<0.05$) between the means of three independent replicates.

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

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

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

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