Enzyme-based strategies for inhibiting medically relevant biofilms: applications in healthcare and food safety

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Abstract: Biofilms are complex communities of microorganisms enclosed in a self-produced matrix of extracellular polymeric substances consisting of polysaccharides, proteins, lipids and extracellular DNA. These biofilms can form on a variety of surfaces and pose a major challenge in both the medical and industrial sectors as they can harbor pathogenic microorganisms. In healthcare, biofilms form on hospital surfaces, medical devices, patient tissue and implants and contribute to persistent infections that are difficult to treat. In the food industry, biofilms on processing equipment and food matrices can also lead to contamination and pose a serious threat to public health through foodborne diseases. The bacteria embedded in biofilms are much more tolerant to antimicrobial treatments than their planktonic counterparts, necessitating the development of new strategies to combat biofilm-associated infections and contamination. As biofilms mature, they become even more resistant to conventional treatments, making prevention strategies particularly important. This review focuses on enzyme-based strategies that have been developed over the last decade to inhibit biofilm formation. Key approaches such as disruption of microbial signaling pathways and degradation of biofilm matrix components are highlighted, offering promising ways to prevent biofilm-related problems in both medicine and industry.

Keywords: biofilm, prevention, enzymes, healthcare, food-industry

1. Introduction

Biofilms are complex communities of microbes embedded in a self-produced matrix of extracellular polymeric substances (EPS) (Flemming *et al.*, 2016). They are the predominant life forms of microorganisms and can be found in almost any environment (Flemming & Wuertz, 2019). They form on rocks, soils, plants and animals surfaces, in extreme environments such as hot springs, and on many man-made surfaces such as pipes and sinks.

While beneficial in some industrial settings (Philipp *et al.*, 2024), biofilms pose a major challenge to human and animal health, food safety and water supply (Cámara *et al.*, 2022; Sentenac *et al.*, 2022).

Medically relevant biofilms can develop on both biotic and abiotic surfaces (Fig. 1). They form on hospital surfaces, medical devices, patient tissues, and implants, as well as other non-medical surfaces (Percival, Suleman, *et al.*, 2015). In addition, biofilms can exist as unattached aggregates and contaminate medical equipment, industrial facilities, and food processing plants. In food processing plants, biofilms can harbor pathogenic microorganisms that contaminate food products and pose a serious risk to food safety and public health.

The biggest health problem associated with biofilms is their high tolerance and resistance to antibiotics. Biofilm-associated infections are often persistent and difficult to treat, requiring prolonged and aggressive therapeutic strategies. Both *in vitro* and *in vivo* studies show that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of clinical antibiotics are generally 10 to 1,000 times higher for biofilm bacteria than those for planktonic bacteria (Høiby *et al.*, 2011). Achieving the effective MBC for biofilm eradication *in vivo* is challenging due to the toxicity and side effects of high-dose antibiotics. This increased biofilm tolerance and even

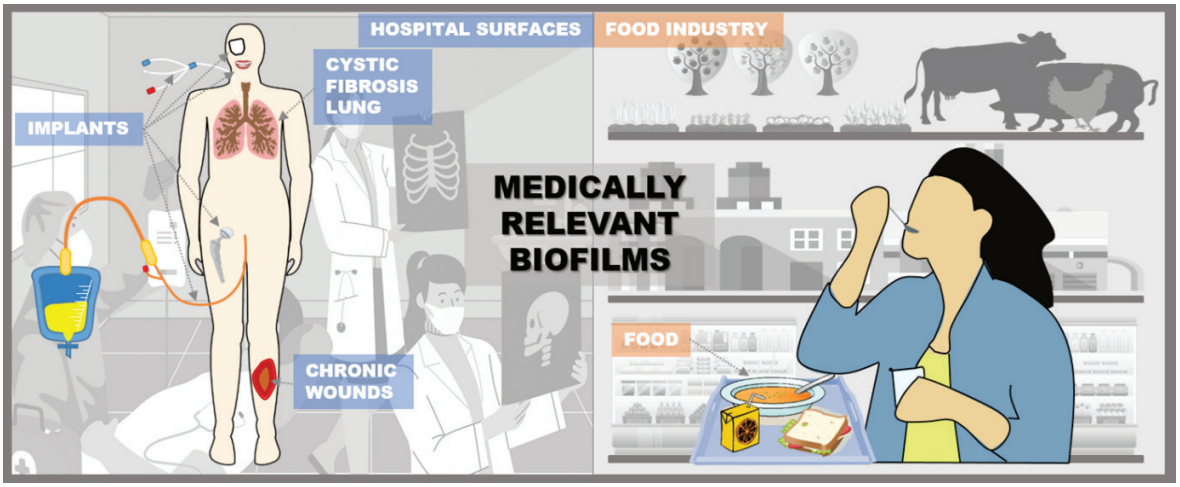

Figure 1. Places of biofilm formation in medicine and the food industry where enzyme-based prevention strategies can be applied. Biofilms often form on abiotic surfaces in hospitals and food processing plants, where they can be inhibited by immobilizing enzymes on these surfaces or by incorporating them into disinfection treatments. In the human body, enzymes can prevent the formation of biofilms when immobilized on wound dressings and various implants or applied in soluble form. Food can be protected by enzyme-functionalized packaging or preservative coatings.

resistance can be attributed to several factors (Ciofu *et al.*, 2022). The EPS matrix, which consists of polysaccharides, proteins, lipids and extracellular DNA (eDNA) (Hobley *et al.*, 2015) inhibits the diffusion of antimicrobial agents and thus reduces their effective concentration within the biofilm (Powell *et al.*, 2021). In addition, the matrix can chemically inactivate some antimicrobial agents through complex formation, enzymatic degradation, or sacrificial reactions (Singh *et al.*, 2017). However, the matrix alone is not the only factor; the slow growth rates of cells in biofilms also contribute to their tolerance. Cells in biofilms often enter a stationary phase or a viable-but-nonculturable state (VBNC), which makes them less susceptible to antibiotics that target actively dividing cells (Ayrapetyan *et al.*, 2018; Stewart & Franklin, 2008). Moreover, the biofilm environment facilitates horizontal gene transfer, a process in which bacteria exchange genetic material (Metzger *et al.*, 2022). This is particularly efficient in biofilms due to the high cell density and close proximity (Mah, 2012).

Due to the low sensitivity of biofilm bacteria to antibiotics, the treatment of infections involving biofilms is often difficult and frequently ineffective. While young biofilms are easier to eradicate, mature biofilms are particularly resistant to treatment, so early diagnosis is necessary for successful intervention (Hengzhuang *et al.*, 2011). However, most clinical biofilm infections are diagnosed at a mature stage, making them difficult to eradicate with antibiotics and often requiring chronic suppressive therapy or management of recurrence (Høiby *et al.*, 2011; Wu *et al.*, 2015).

Research into the formation, structure and function of biofilms is progressing and aims to develop innovative strategies to combat biofilms. To develop effective approaches to biofilm elimination, current research efforts are focused on the discovery of new antimicrobial agents and surface coatings that can prevent biofilm formation and reduce the burden of biofilm-associated infections and contamination. To prevent biofilm formation, it is critical to prevent microbial colonization of surfaces, as colonization is the first step in biofilm development and the moment when the biofilm is most susceptible (P. Gupta *et al.*, 2016). This review focuses on inhibition strategies for medically relevant biofilms based on enzymes that have been investigated over the last decade.

2. Biofilm formation

The formation of biofilms is a complex process consisting of three main phases: Attachment to the surface, maturation and dispersal (Sauer *et al.*, 2022). In the attachment phase, the planktonic cells come into contact with a surface, which can be either biotic (e.g. skin or bone) or abiotic (e.g. medical devices such as urinary catheters or artificial joints). In tissue infections, the bacteria adhere to each other and form aggregates. This initial adhesion is weak and reversible and is influenced by surface properties and environmental conditions. With the help of pili and fimbriae, the adhesion of the bacteria to the surface then becomes much stronger. Once the bacteria begin to produce EPS, which enables the cells to adhere strongly to each other and/or to a surface, the adhesion becomes irreversible.

During the maturation phase, the attached bacteria begin to multiply and form microcolonies. EPS production increases, forming a scaffold that holds

the growing community together and develops into three-dimensional structures. As the biofilm continues to grow and mature, it becomes more complex and structured, creating a microenvironment characterized by nutrient and oxygen gradients. This leads to physiological heterogeneity, with fast-growing cells on the surface and slow-growing, tolerant cells in the deeper layers. As a result, the biofilm becomes more resistant to antibiotics and environmental stress during this phase. Finally, in the dispersion phase, cells or cell clusters detach from the biofilm to colonize new surfaces. This process is triggered by environmental changes or a lack of nutrients.

Biofilm formation depends on various regulatory signals, including messengers such as cyclic di-guanylate (c-di-GMP), small non-coding regulatory RNA (sRNA) and small molecules called autoinducers (AIs) via quorum sensing (QS). The switch between planktonic and biofilm lifestyle is controlled by c-di-GMP (Römling *et al.*, 2013). High c-di-GMP concentrations trigger the production of adhesins and matrix polysaccharides and inhibit various types of motility, thereby promoting biofilm formation. Conversely, low levels of c-di-GMP reduce the production of adhesins and exopolysaccharides and increase bacterial motility, leading to biofilm dispersal (for a comprehensive overview see Valentini & Filloux, 2016).

sRNAs regulate biofilm formation by modulating gene expression, often at the post-transcriptional level (Taylor *et al.*, 2017; Thomason *et al.*, 2012). sRNAs help bacteria adapt to environmental stresses such as nutrient starvation and antimicrobial agents by regulating stress response genes, thereby improving biofilm stability and resistance. In addition, sRNAs influence the synthesis of extracellular polymeric substances (EPS), which are important components of the biofilm matrix, and regulate genes involved in biofilm dispersal, allowing the bacteria to return to a planktonic state when conditions change. Through these mechanisms, sRNAs control the complex processes underlying biofilm formation, maintenance and dispersal. The role of sRNA in biofilm formation has recently been reviewed (Mitra & Mukhopadhyay, 2023)

QS is a cell-to-cell communication system that enables bacteria to make collective decisions based on cell density and behave like a multicellular organism. A QS system consists of AIs, synthases that produce the AI, and a receptor/transcriptional regulator that recognizes the signal. When the AI binds to its receptor, it activates gene transcription, including AI biosynthesis, creating a positive feedback loop. At low cell density, AI levels are low and the receptor is only marginally active. With increasing cell density, the AI concentration reaches a threshold value that leads to complete activation of the receptor and upregulation of the target genes. Gram-positive and Gram-negative bacteria use different types of AIs. Gram-positive bacteria produce short oligopeptides as AIs, while signaling molecules of Gram-negative bacteria are small molecules such as acyl homoserine lactones (AHLs), alkylquinolones, thiazole compounds, α-hydroxyketones and diffusible signaling factors (fatty acid-like compounds) (Senerovic *et al.*, 2020).

Some bacteria can use a single type of AI, while other species use QS system consisting of multiple pathways regulated by structurally different AIs. *Pseudomonas aeruginosa*, for example, uses four QS signaling pathways known as Las, Rhl, Pseudomonas Qunolone Signaling (PQS) and IQS (Papenfort & Bassler, 2016). The QS signaling pathways alone or interconnected regulate genes commonly associated with pathogenicity and influence virulence factor production, biofilm formation and various types of motility (Rutherford & Bassler, 2012).

The QS play a species-specific role in biofilm formation and influence the structural development and stabilization of the biofilm. In Gram-negative bacteria (e.g. *P. aeruginosa* and *Burkholderia cenocepacia*), QS is involved in the release of significant amounts of eDNA at later stages of biofilm development by autolysis of a bacterial subpopulation (Allesen-Holm *et al.*, 2006; Pakkulnan *et al.*, 2019). In *P. aeruginosa*, QS also regulates the synthesis of rhamnolipids, which are crucial for the late stages of biofilm development. Rhamnolipids maintain channels in mushroom-shaped biofilm structures, ensuring the proper distribution of nutrients and oxygen and the removal of waste products (Davey *et al.*, 2003). Overproduction of rhamnolipids causes the biofilm to detach and spread (Boles *et al.*, 2005). In addition, QS controls the production of LecA and LecB lectins (Winzer *et al.*, 2000) and siderophores such as pyoverdin and pyochelin (Popat *et al.*, 2017). These siderophores are involved in iron metabolism, with both low and high concentrations of iron inhibiting biofilm formation (Banin *et al.*, 2005). In biofilms, AIs can reach much higher concentrations compared to environment of free-living organisms, which increases their effectiveness. The biofilm matrix itself can bind to and concentrate signaling molecules, enabling more effective QS (Keller & Surette, 2006).

3. Strategies to prevent medically relevant biofilms

Biofilm-forming microorganisms are responsible for 60-80% of human infections, particularly in healthcare settings, with the most problematic being methicillin-resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa*. These bacteria can form biofilms on medical devices like catheters and prosthetic joints, as well

as in the lungs of cystic fibrosis patients and chronic wounds, leading to persistent, hard-to-treat infections. Other notable biofilm formers include uropathogenic *Escherichia coli*, which can cause chronic urinary tract infections, *Enterococcus faecalis*, a common cause of hospital-acquired infections such as endocarditis, *Klebsiella pneumoniae*, which poses serious risks to immunocompromised patients, and *Streptococcus mutans*, a major contributor to dental plaque and caries. The presence of biofilms enhances bacterial resistance to the immune system and antibiotics, complicating treatment strategies for these infections.

The most common foodborne pathogens that form biofilms include *Salmonella* spp. which cause salmonellosis and can adhere to various surfaces in food processing facilities, leading to significant health risks. *E. coli*, especially enterohemorrhagic strains such as O157, can form biofilms on food and food contact surfaces, leading to serious foodborne illness. *Listeria monocytogenes* is notorious for forming biofilms in cold and damp food processing facilities, leading to listeriosis, a serious infection with a high mortality rate. *Campylobacter jejuni*, a major cause of bacterial gastroenteritis, can form biofilms on poultry and other surfaces, contributing to food contamination. *S. aureus* and *Bacillus cereus* can form biofilms on food and surfaces and produce toxins that cause food poisoning. These pathogens pose a major challenge in food processing as they easily adhere to surfaces and form biofilms that resist cleaning and hygiene measures, leading to persistent contamination and food safety issues (Galié *et al.*, 2018).

The strategies to inhibit biofilm formation are based on the two types of biofilm inhibitors with different modes of action:

1) Bactericidal agents: these molecules prevent biofilm formation by inhibiting bacterial growth. Common bactericidal biofilm inhibitors include novel small molecules and structural analogs of clinical antibiotics (Blasco *et al.*, 2024; Kokot *et al.*, 2023), metal complexes (Glišić *et al.*, 2016; Rinehart *et al.*, 2023; Savić *et al.*, 2016; Sovari *et al.*, 2021), nanoparticles (A. Gupta *et al.*, 2018), plant derived molecules, extracts and essential oils (Gómez-Sequeda *et al.*, 2020; Sánchez *et al.*, 2016; Zizovic *et al.*, 2018), antimicrobial peptides (Klubthawee *et al.*, 2023), bacteriophages (Vukotic *et al.*, 2020), and probiotics that produce antimicrobial metabolites (Al-Shamiri *et al.*, 2023; Lee *et al.*, 2021). While they are effective in the early stages of infection, prolonged use of bactericidal agents can lead to resistance that compromises the effectiveness of treatment.

2) Antivirulence molecules, in contrast to bactericidal agents, specifically target the virulence properties of pathogens, including biofilm formation, without interfering with bacterial growth. This makes antivirulence strategies particularly beneficial for the treatment of infections that are prone to resistance, as they pose a lower risk of resistance development.

Effective prevention of biofilm formation with antivirulence agents can be achieved by targeting different stages of the biofilm formation process.

a) Bacterial adhesion to surfaces can be interrupted by the use of biosurfactants (Aleksic *et al.*, 2017; Amirinejad *et al.*, 2022; Sambanthamoorthy *et al.*, 2014) such as glycolipids, rhamnolipids, lipopeptides, polysaccharide-protein complexes, phospholipids or fatty acids, antibodies targeting adhesion proteins such as pili or fimbriae (de Freitas *et al.*, 2021; D. Sun *et al.*, 2005), or chelators that can remove cations (Fe, Ca^{2+} and Mg^{2+}) that play a role in microbial adhesion and biofilm formation (Abraham *et al.*, 2012; O'May *et al.*, 2009).

b) Inhibition of QS and c-di-GMP signaling pathways

Quorum sensing can be disrupted at multiple points within the signaling pathway. Approaches include inhibiting the synthesis of autoinducers (AIs) using small molecules that target synthases (Chang *et al.*, 2014), using quorum quenching (QQ) enzymes to hydrolyze AIs to reduce their concentration (Djokic *et al.*, 2022; Koch *et al.*, 2014; Malešević *et al.*, 2020; Pustelny *et al.*, 2009), and blocking the interaction between AIs and their receptors by competitive inhibition with naturally derived, synthetic or semi-synthetic molecules that are structurally similar to natural AIs (Aleksic *et al.*, 2018, 2019; Aleksić *et al.*, 2017; Pekmezovic *et al.*, 2016).

c-di-GMP signaling can be modulated by small molecule inhibitors of c-di-GMP synthesis (Andersen *et al.*, 2021; Chua *et al.*, 2015) or by direct reduction of c-di-GMP levels with c-di-GMP sequestering peptides (Hee *et al.*, 2020).

3) Inhibition of biofilm maturation can be achieved by blocking the biosynthetic pathways of extracellular polymeric substances (EPS) with small molecules (Razvi *et al.*, 2023) or by hydrolyzing EPS components using specific enzymes (Atanaskovic *et al.*, 2024; Baker *et al.*, 2016; Banar *et al.*, 2016; Lim *et al.*, 2019).

Biofilm inhibitors can be applied directly by applying the agents to the site of infection or indirectly by functionalizing materials such as surfaces, medical devices and wound dressings to prevent biofilm formation.

4. Enzyme-based strategies to inhibit biofilm formation

Enzyme -based strategies offer a targeted method to combat biofilms as they are highly specific for biofilm

components or signaling molecules, which helps to reduce off-target effects. The use of anti-biofilm enzymes carries no risk of resistance development as they act on extracellular compounds and not directly on bacterial cells. As they do not have a bactericidal effect, they should be used in combination with antibacterial agents to achieve maximum efficacy. In combination with antibiotics, enzymes can increase the efficacy of drugs by breaking down biofilm barriers or inhibiting bacterial virulence factors (Borges *et al.*, 2020). However, enzyme-based therapies face challenges such as the instability of enzymes under certain conditions, high production costs and the need to combine them with other treatments (antibiotics or disinfectants). There is also a risk of immune reactions against the enzymes (Ghosh *et al.*, 2019).

Enzyme-based inhibition of biofilm formation is achieved either by disrupting signaling cascades within and between microorganisms or by interfering with the synthesis of biofilm matrix components (Nahar *et al.*, 2018). Here we discussed different types of enzymes including QQ enzymes, cyclic di-GMP degrading enzymes, exopolysaccharide degrading enzymes, proteolytic enzymes, oxidative enzymes, deoxyribonucleases, and lipolytic enzymes with potential to prevent formation of various medically relevant biofilms. However, most research in this field has focused on QQ enzymes and glucoside hydrolases, with *P. aeruginosa* being the most frequently studied pathogen (Table 1). This review presents the current state of the field and identifies research gaps that could be filled by studying other enzyme types and a broader range of pathogens.

Table 1. Summary of enzymes with proposed application in biofilm prevention, described in last 10 years

Enzyme	Type of enzyme	Mechanism	Substrate	Target pathogens	Reference
YtnP	Lactonase	Quorum-quenching enzyme	N-acyl homoserine lactones	*P. aeruginosa*	(X. Sun *et al.*, 2021)
YtnP-ZP1				*P. aeruginosa*	(Djokic *et al.*, 2022)
AidHA147G				*P. aeruginosa*	(Zhang *et al.*, 2023)
Ahl-1				*P. aeruginosa*	(Sakr *et al.*, 2021)
PvdQ	Acylase			*P. aeruginosa*	(Vogel *et al.*, 2020)
Acylase I				*P. aeruginosa* *E. coli*	(Grover *et al.*, 2016; K. Ivanova *et al.*, 2015)
PF2571				*A. veronii*	(D. Wang *et al.*, 2022, 2023)
PslG	Glycoside-hydrolase	Matrix degrading enzyme	Psl exopolysaccharide	*P. aeruginosa*	(Asker *et al.*, 2018, 2021; Zhang *et al.*, 2023)
PelA			Pel exopolysaccharide	*P. aeruginosa*	(Szymańska *et al.*, 2020)
α-amylase			Exopolysaccharides	*P. aeruginosa E. coli* *S. aureus*	(A. Ivanova *et al.*, 2021; K. Ivanova *et al.*, 2015)
Dextranase			dextran	*S. mutans*	(Juntarachot *et al.*, 2020)
Lysozyme			Peptidoglycan, exopolysaccharides	*P. aeruginosa* *S. aureus*	(Kiani *et al.*, 2023)
β glucosidase BglB-BG28			Cellulose	*S. enterica* *E. coli*	(Atanaskovic *et al.*, 2024)
Predicted glycosyl hydrolase			Exopolysaccharides	*S. enterica* *E. coli* *L. monocitogenes*	(Mayton *et al.*, 2021)
DNase I	Deoxyribonuclease		eDNA	*P. aeruginosa* *S. aureus* *S. mutans*	(Alves *et al.*, 2016; Ye *et al.*, 2017)
Papain	Proteinase		Proteins	*Acinetobacter sp.* *S. aureus* *E. coli*	(C. Manohar, 2014; C. M. Manohar *et al.*, 2015; Prabhawathi *et al.*, 2014)
Flavorzyme	Peptidase complex	Protein degrading enzyme		*S. enterica* *E. coli*	(Nahar *et al.*, 2021)

4.1 Application of anti-biofilm enzymes in healthcare

Biofilm formation on hospital surfaces poses a significant threat to patient safety and health outcomes. Biofilms can form on the surfaces of medical devices, frequently touched areas, and medical instruments (Assefa & Amare, 2022). These biofilms are notoriously difficult to eliminate and are associated with a high prevalence of hospital-acquired infections, accounting for over 65% of these cases (Preda & Săndulescu, 2019). The presence of biofilms highlights the urgent need for effective cleaning and disinfection protocols to reduce the risk of infection transmission in healthcare facilities.

Contamination of water lines poses a significant risk of microbial transfer to hospital surfaces, contributing to the potential spread of infections among patients and healthcare providers (Suleyman *et al.*, 2018). Sun and colleagues developed a water filtration system to simulate biofilm contamination on water filters in dental unit water lines, highlighting the health risks these biofilms pose to both patients and dentists (X. Sun *et al.*, 2021). They tested the antifouling activity of YtnP lactonase by applying it to *P. aeruginosa* inoculated water, which was continuously flowed through the filter membrane. Successful degradation of N-acylhomoserine lactones inhibited EPS production and reduced biofilm formation, as well as virulence factors production such as pyocyanin and rhamnolipids, suggesting its potential as a novel disinfectant for dental units.

In a different approach, Asker and colleagues used surface adsorption and covalent attachment techniques to immobilize PslGh on glass, polydimethylsiloxane (PDMS), and polystyrene (PS) surfaces (Asker *et al.*, 2018). It was achieved by covalently attaching a Psl-specific glycoside hydrolase (PslGh) to various chemically distinct surfaces using amine functionalization (APTMS) and glutaraldehyde binding, which effectively inhibited *P. aeruginosa* colonization. Over a period of 8 days, PslGh-modified surfaces showed a significant reduction in surface attachment and biofilm formation by *P. aeruginosa*, achieving a reduction of surface-associated bacteria by approximately 99.9% (approximately 3-log) compared to control surfaces that were either untreated or treated with an inactive enzyme.

Biofilms are not limited to colonizing abiotic surfaces, they can also thrive on biotic surfaces. These microbial communities can develop in the tissues of patients and lead to chronic infections that are difficult to treat. Some of the most common infections associated with biofilms are those that occur in chronic wounds, in the airways of cystic fibrosis patients, and in association with medical implants (Del Pozo, 2018).

4.1.1 Chronic wound-associated biofilms

When the skin, our natural protective barrier, is compromised, it leaves the body vulnerable to microorganisms from the patient's own flora or the external environment. Initially, the host's immune system can control these microbes, but if they adhere to the wound surface and begin to multiply, a biofilm can form. As the biofilm matures, it becomes increasingly resistant to both the immune response and antimicrobial treatments, complicating wound care and raising the risk of chronic infection (Percival, *et al.*, 2015). Given that patients with chronic wounds often have underlying medical conditions that compromise their immune systems (Falanga *et al.*, 2022), the majority of these wounds (~80%) become colonized with biofilms (Malone *et al.*, 2017). Most of the chronic wounds biofilms are polymicrobial, but when single-species biofilms occur, they are most likely caused by *Pseudomonas* or *Staphylococcus* genera (Wolcott *et al.*, 2016). Another condition that harbors a fruitful environment for biofilm development is burn injury. It makes a patient immunocompromised and susceptible to opportunistic pathogens such are *P. aeruginosa*, *Acinetobacter baumanii* and *S. aureus* (Maslova *et al.*, 2021). The economic burden of wound management on the UK's NHS in 2017/2018 was £8.3 billion (Guest *et al.*, 2020). Venous ulcers affect up to 1% of the US population, and diabetic foot ulcers impact 15-25% of diabetics (Richmond *et al.*, 2013). Biofilm-associated chronic wounds may lead to serious consequences such as sepsis or amputation (Eriksson *et al.*, 2022). Diabetics face a tenfold higher incidence of lower limb amputation and a 5-year mortality rate of 40% after ulceration (Jupiter *et al.*, 2015). Additionally, 75% of all deaths in burned patients are caused by wound infection (Maslova *et al.*, 2021).

In view of all these statistics and clearly identified risk groups, biofilm prevention is an important step in wound care. Enzyme-functionalized dressings are an attractive strategy for biofilm control in open wounds (Roy *et al.*, 2017). Over the past 10 years, researchers have used QQ enzymes lactonases and *P. aeruginosa*-specific glycoside hydrolases (PslG and PelA) to test this theory, focusing mainly on *P. aeruginosa* as a causative agent of wound infection. Zhang *et al.* conducted an *in vivo* study in rats with burns and surface infections using a combined enzyme therapy of N-acylhomoserine lactonase AidHA147G, glycoside hydrolase PslG and tobramycin (Zhang *et al.*, 2023). This therapy targets quorum sensing molecules and the Psl exopolysaccharide of *P. aeruginosa* and was administered once daily between the surgical dressing and the wound surface. The combined enzymes enhanced the antibiotic effect, leading to a reduction in inflammation, tissue damage and bacterial counts.

In addition, the combined enzymes showed certain therapeutic effects even without the use of antibiotics (Zhang *et al.*, 2023). A similar effect was observed in infected zebrafish tail wounds (Djokic *et al.*, 2022). *P. aeruginosa* infection was cleared within 2 days by using YtnP-ZP1 lactonase in combination with tobramycin. Another administration technique was used by dissolving the lactonase Ahl-1 in 5% carboxymethylcellulose hydrogel and applying it in a burnt mouse model (Sakr *et al.*, 2021). The survival rate was 100 compared to a control group in which a survival rate of 20 was observed.

By binding enzymes to solid supports or encapsulating them in matrices, researchers and manufacturers are harnessing the catalytic power of enzymes while overcoming the challenges associated with their stability and reusability (Khan, 2021). In addition to immobilization in hydrogels, wound care can also benefit from enzymes immobilized on dressing material. Bacterial nanocellulose (BC) has been shown to be an ultrafine network for wound dressings as it provides a moist, biocompatible environment while acting as an effective physical barrier against external infection (Bilal & Iqbal, 2019). Szymańska *et al.* immobilized the hydrolytic domain of PelA (PelAh) on BC membranes by physical adsorption (Szymańska *et al.*, 2020). This affected biofilm formation by disrupting the integrity of the biofilm matrix, making it easier for adherent cells to detach from the BC surface. They suggest that the developed method could be improved by incorporating specific glycoside hydrolases targeting other components of the biofilm matrix, which could create a synergistic effect leading to more effective biofilm eradication and reducing the necessary dosage of additional antimicrobial chemotherapeutics.

4.1.2 Cystic fibrosis-associated biofilms

Cystic fibrosis (CF) is a genetic disorder that primarily affects the lungs and digestive system and is characterized by the production of thick, sticky mucus that obstructs the airways and leads to severe respiratory complications. Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which disrupt ion transport and lead to dehydrated mucus (Cutting, 2015). This mucus accumulation creates an environment that favors chronic bacterial infections, particularly with *P. aeruginos*a and *S. aureus*, and leads to persistent inflammation and lung damage (Cohen & Prince, 2012). Recombinant human DNase, commonly known as Dornase alfa, is a treatment option for cystic fibrosis patients that is mainly used to improve lung function and reduce the viscosity of mucus in the airways. This enzyme cleaves extracellular DNA that accumulates in the lungs due to inflammation and infection, especially from neutrophils (Shak, 1995), but also from bacterial biofilm matrix (Z. Wang *et al.*, 2024). Although clinical studies up to 2006 showed that the consistent use of DNase can significantly reduce the occurrence of pulmonary exacerbations, slow down the deterioration of lung function and prevent bacterial infections (Frederiksen *et al.*, 2006), no other enzyme with comparable properties has been used to date.

4.1.3 Medical implant-associated biofilms

Medical implants are artificial devices inserted into the body for diagnostic, therapeutic, or rehabilitative purposes. Most commonly used medical devices are neurological, dental, cardiovascular, gastrointestinal, urological, intravascular, orthopedic implants, contact lenses, breast implants, IUDs and penile implants (Caldara *et al.*, 2022). Unfortunately, device-associated infections, often associated with biofilm formation, remain a significant global health problem with serious clinical and economic implications (Percival, Suleman, *et al.*, 2015). *Staphylococcus* species are the most commonly isolated bacteria on most medical devices (Caldara *et al.*, 2022). Gastrointestinal implants have the greatest diversity of colonizing microorganisms, including a high prevalence of Enterococci, Enterobacteriaceae, *Bacillus* spp. and Streptococci, in addition to *Staphylococcus* spp. (Dautle *et al.*, 2003). Urological implants and intravascular devices are frequently colonized by *E. faecalis*, *P. aeruginosa* and *K. pneumoniae* (Holá *et al.*, 2010; Schulze *et al.*, 2021). In addition to these bacteria, urological implants are also frequently colonized with *E. coli*, *Proteus mirabilis* and *Enterobacter* sp. (Holá *et al.*, 2010). Dental implants in particular harbor the most distinct microbial communities, including *Fusobacterium* spp, *Porphyromonas gingivalis*, *Prevotella* spp., *Selenomonas* spp., *Staphylococcus* spp. and *Streptococcus* spp .(Caldara *et al.*, 2022).

As far as the functionalization of implant surfaces with anti-biofilm enzymes is concerned, most work in the last 10 years has been carried out on urinary and venous catheters. Researchers in this field have used amylase (broad-spectrum glycoside hydrolase), acylase (QQ enzyme), PslG (Psl-specific glycoside hydrolase) and DNase to functionalize catheter surfaces and prevent biofilm formation of various pathogens such as *E. coli*, *S. aureus* and *P. aeruginosa*.

Catheter-associated urinary tract infections (CAUTIs) account for over 40 % of hospital-acquired infections and more than 80 % of all urinary tract infections and represent a global health problem (Milo *et al.*, 2019). These infections increase mortality and morbidity, prolong hospitalization, increase healthcare costs, require prolonged antibiotic therapy and increase the risk of antibiotic resistance (Tenke *et al.*, 2017). To

prolong the lifespan of urinary catheters, reduce the incidence of CAUTIs and curb antibiotic resistance, Ivanova and colleagues proposed an innovative anti-biofilm coating. They combined antibacterial zinc oxide nanoparticles (ZnO NPs) with the exopolysaccharide-degrading enzyme amylase and applied them to silicone urinary catheters in a one-step sonochemical process. This nano-enhanced coating successfully inhibited biofilm formation by *E. coli* and *S. aureus* by 80 % and 60 %, respectively, for up to seven days in an *in vitro* catheter bladder model with artificial urine recirculation. *In vivo* experiments in a rabbit model confirmed the results that coated catheters led to a lower incidence of bacteriuria and delayed the early onset of CAUTIs compared to untreated silicone catheters (Ivanova *et al.*, 2021). Vogel and coworkers used PvdQ acylase to create a QQ surface on polydimethylsiloxane (PDMS) using electrostatic interactions. The immobilized acylase maintained its activity after coating and showed a 6-fold reduction of the AI level (3-oxo-C12) and a significant reduction of a *P. aeruginosa* biofilm on a coated PDMS in a biosensor setup compared to the same untreated material (Vogel *et al.*, 2020). Acylase derived from *Aspergillus melleus* was immobilized on biomedical polyurethane coatings using a multi-point covalent immobilization technique (Grover *et al.*, 2016). These acylase-containing coatings showed enzymatic activity and effectively catalyzed the hydrolysis of AIs such as N-butyryl-L-homoserine lactone (C4-LHL), N-hexanoyl-L-homoserine lactone (C6-LHL) and N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-LHL). In biofilm inhibition tests, the immobilization of the acylase led to a reduction in biofilm formation by *P. aeruginosa* of about 60%. Scanning electron microscopy also showed that the acylase- containing coatings had significantly fewer bacterial cells than the control coatings without acylase. Importantly, the acylase-embedded coatings maintained 90% of their activity after being stored dry at 37 °C for 7 days and exhibited greater stability than the free enzyme under physiological conditions, including in artificial urine. A combination of amylase and acylase was utilized to coat silicone urinary catheters using a layer-by-layer deposition technique (Ivanova *et al.*, 2015). In a static biofilm assay, catheters coated with the acylase in the top layer showed a 60% reduction of biofilms formed either by only *P. aeruginosa* or both *P. aeruginosa* and *E. coli*. The comparable results were observed in the physical model of a catheterized human bladder, while *in vivo* experiments on catheterized rabbits showed biofilm inhibition only at certain catheter segments depending on the position in the animal body.

Central venous catheters (CVCs) are also highly susceptible to microbial colonization and biofilm formation, posing a significant risk for hospital-acquired infections. The average incidence of CVC-associated bloodstream infections in the US is 5.3 per 1000 catheter days in the ICU, which equates to approximately 80,000 cases per year (Gominet *et al.*, 2017). Asker and colleagues showed that PslGh can be uniformly immobilized on the inner surfaces of catheter tubing made of medical grade polyethylene (PE-100), polyurethane and polydimethylsiloxane (silicone) (Asker *et al.*, 2021). PslGh was covalently bound to the activated surface. Under dynamic flow culture conditions, *P. aeruginosa* colonization and biofilm formation showed a 3-log reduction in bacterial counts in the first 11 days and a 2-log reduction by day 14 for PslGh-modified PE-100 catheters compared to untreated controls. This was transferred to a rat infection model. PslGh-modified PE-100 catheters showed an approximate 1.5-log reduction in colonization of the clinical *P. aeruginosa* strain after 24 hours. In addition, a bifunctional coating with antimicrobial and anti-biofilm properties was used against both *S. aureus* and *P. aeruginosa* (Alves *et al.*, 2016). Using polydopamine coating technology, they co-immobilized the antimicrobial lipopeptide Palm and the enzyme DNase I, which is able to degrade extracellular DNA from the biofilm matrix of both pathogens.

Deoxyribonuclease I (DNase I) was immobilized on a titanium surface (Ti) using dopamine as a linking agent (Ye *et al.*, 2017). Titanium is widely used in the medical field due to its exceptional properties such as biocompatibility, corrosion resistance and strength to weight ratio. Since the 1950s, it has been the material of choice for a variety of applications, such as cardiovascular devices, orthopedic and dental implants. Titanium's ability to integrate with bone, known as osseointegration, allows it to bond effectively with human tissue and makes it ideal for joint replacement and fracture fixation (Balazic *et al.*, 2007). The resulting DNase-I coating showed significant efficacy in preventing adhesion and biofilm formation of *S. mutans* and *S. aureus* over a 24-hour period (Ye *et al.*, 2017). *S. mutans* is the most common pathogen in dental plaque, a form of biofilm that forms on surfaces in the oral cavity. Juntarachot *et al.* developed a toothpaste with dextranase encapsulated in alginate beads with an effective dose of 4.5 units/g (Juntarachot *et al.*, 2020). Brushing movements mechanically released the dextranase from the beads, which remained active for a prolonged period after brushing and are intended to inhibit biofilm formation by dissolving dextrans from the biofilm matrix.

Bandage contact lenses tend to accumulate bacterial biofilms during wear, with the two most common pathogens being *P. aeruginosa* and *S. aureus* (Zhu *et al.*, 2019). Since lysozyme occurs naturally in tears,

Kiani *et al.* investigated the effect of lysozyme on biofilm formation on bandage contact lenses by these two pathogens (Kiani *et al.*, 2023). Lysozyme is a glycoside hydrolase that primarily targets glycosidic bonds in the peptidoglycan, but is also capable of destroying the biofilm matrix. It was physically adsorbed to the silicone hydrogel bandage contact lenses and inhibited the biofilm formation of 38.3% and 62.7% of *P. aeruginosa* and *S. aureus*, respectively. These results suggested that bandage contact lenses functionalized with lysozyme may reduce the risk of ocular infection after ocular surgery.

4.2. Application of anti-biofilm enzymes in food industry

Pathogenic biofilms in the food industry pose a significant health risk and an economic challenge. The biofilms formed by various foodborne pathogens such as *B. cereus*, *S. aureus*, *L. monocytogenes*, *E. coli* and *Salmonella enterica*, can develop on food contact surfaces and equipment and form a persistent reservoir of contamination (Galié *et al.*, 2018). The presence of biofilms not only increases the likelihood of foodborne disease outbreaks, which account for around 60% of such incidents, but also complicates cleaning and hygiene measures due to their increased resistance to antimicrobial agents and physical removal methods (Liu *et al.*, 2023). In the last 10 years, scientists have used anti-biofilm enzymes (β-glucosidase, flavorzymes, predicted glycoside hydrolase) in surface disinfection protocols targeting the structural components of biofilm. Atanaskovic and coworkers demonstrated the inhibitory effect of the β-glucosidase BglB-BG28 on *Salmonella* and *E. coli* biofilms on plastic, glass and metal surfaces (Atanaskovic *et al.*, 2024). The enzyme targeted the synthesis process of cellulose fibers, one of the main components of *Salmonella* and *E. coli* biofilms. It was compatible with non-ionic detergents and showed a synergistic effect when subsequently treated with Oxicid S, a disinfectant commonly used in food processing plants and animal enclosures. The biofilm formation of *Salmonella* and *E. coli* was also successfully inhibited using a commercial peptidase, Flavorzyme (Nahar *et al.*, 2021). The suggested mechanism is the inhibition of bacterial self-defense mechanisms by interfering with cellular proteins. Mayton et al had success in inhibiting biofilm formation, not only of Gram-negative *Salmonella* and *E. coli*, but also Gram-positive *L. monocytogenes* (Mayton *et al.*, 2021). They used a parallel-plate flow chamber to directly observe the adhesion and detachment of the cells from the surface with a fluorescence microscope, thus simulating the real rinsing process. The enzyme was a predicted glycosyl hydrolase, and they proposed the degradation of exopolysaccharides as the mechanism.

4.2.1 Food-associated biofilms

Recent studies have explored innovative approaches to improve food safety through antimicrobial packaging to address the important issue of food contamination during processing, which contributes to spoilage and foodborne illness. The most commonly used enzyme for food packaging functionalization in the last 10 years has been papain, a proteolytic enzyme capable of destroying the structural components of biofilm. One such approach is the use of papain immobilized on polyurethane with the help of glutaraldehyde. This food packaging significantly reduced *S. aureus* biofilm and bacterial contamination of cottage cheese stored at 4°C for 7 days and showed superior antimicrobial activity compared to unmodified polymer films (Manohar, 2014). Papain was also immobilized on other materials, including low-density polyethylene (LDPE), high density polyethylene (HDPE), linear low density polyethylene (LLDPE) and polycaprolactam (PCL) using curcumin as a photocrosslinker (Manohar *et al.*, 2015). The immobilized enzyme retained more than 90% of its activity after 30 days, with LLDPE showing the best biofilm properties against *Acinetobacter* sp. and *S. aureus*. This method effectively reduced microbial contamination of meat wrapped in modified LDPE and stored at 4°C for 7 days. In addition, papain-functionalized polycaprolactam was tested as a packaging material against *E. coli* biofilm. This material effectively inhibited biofilm growth and showed a drastic reduction in bacterial counts over 30 days (Prabhawathi *et al.*, 2014). Another approach is to combat food spoilage through targeted bacterial QS. Wang *et al.* investigated the changes in the microbiota of red bream fillets during cold storage and found that the microbial diversity shifted in favor of *Aeromonas veronii* on the fourth day of storage (Wang *et al.*, 2022). They proposed a strategy to alter the microbiota using the QQ acylase PF2571. The enzyme effectively inhibited spoilage-related QS factors, including biofilm formation, motility, protease, lipase and alginate production. The treated fillets maintained their quality for a longer period of time than the untreated controls, highlighting the potential for extending the shelf life of fish and fishery products. Next, Wang and colleagues developed a tamarind polysaccharide-polyvinyl alcohol hydrogel for seafood preservation (Wang *et al.*, 2023). This hydrogel exhibited self-healing properties and demonstrated pH-dependent release of the QQ acylase PF2571. *In vivo* tests showed that hydrogel-coated fish fillets had a shelf life extended by more than three days.

5. Conclusion

The application of anti-biofilm enzymes in the healthcare and food industries represents a promising

opportunity to combat the ongoing challenge of biofilm-associated infections and contamination. Biofilms, known for their resilience and contribution to hospital-acquired infections, significantly impact patient safety and healthcare outcomes. The integration of enzymes such as lactonases, acylases, glycoside hydrolases, DNases and proteases into cleaning protocols, medical devices, wound dressings and even food packaging offers a novel and targeted approach to biofilm prevention and elimination. In healthcare, the use of these enzymes to disrupt biofilm formation on medical devices and chronic wounds has shown significant potential. Enzyme-functionalized dressings and coatings on medical implants have demonstrated their ability to reduce bacterial colonization and biofilm formation, thereby reducing the risks of chronic infections and device-related complications. In the food industry, anti-biofilm enzymes have been successfully used to improve surface sanitization and increase food safety through antimicrobial packaging. These enzymes target the structural components of biofilm, reducing the risk of foodborne disease outbreaks and extending the shelf life of perishable goods. The ongoing development and refinement of enzyme-based approaches holds great promise for improving health practices and food safety. However, further research is needed to optimize these applications and ensure their efficacy and stability in different environments. By harnessing the catalytic power of enzymes, we can develop more effective, sustainable solutions to biofilm-related challenges in different sectors.

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