Enzymatic dispersion of biofilms: An emerging biocatalytic avenue to combat biofilm-mediated microbial infections

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Drug resistance by pathogenic microbes has emerged as a matter of great concern to mankind. Microorganisms such as bacteria and fungi employ multiple defense mechanisms against drugs and the host immune system. A major line of microbial defense is the biofilm, which comprises extracellular polymeric substances that are produced by the population of microorganisms. Around 80% of chronic bacterial infections are associated with biofilms. The presence of biofilms can increase the necessity of doses of certain antibiotics up to 1000-fold to combat infection. Thus, there is an urgent need for strategies to eradicate biofilms. Although a few physicochemical methods have been developed to prevent and treat biofilms, these methods have poor efficacy and biocompatibility. In this review, we discuss the existing strategies to combat biofilms and their challenges. Subsequently, we spotlight the potential of enzymes, in particular, polysaccharide degrading enzymes, for biofilm dispersion, which might lead to facile antimicrobial treatment of biofilm-associated infections.

The emergence of multiple drug resistance in pathogenic microbes has led to poor management of bacterial and fungal diseases in both humans and animals. Microorganisms employ multiple defense mechanisms against the host immune system and antimicrobials, which lead to establishment of microbial pathogenesis. Biofilm formation is one of the leading reasons for the emergence of multidrug-resistant bacteria and fungi that affects human health and the world economy severely (1–4). These microorganisms have two distinct modes of survival: motile and sedentary. Biofilms are representative of bacteria and fungi in the sedentary state of their life cycle as opposed to a motile state. Bacteria and fungi have an innate tendency to cling onto both biotic and abiotic surfaces and ensconce themselves in a self-produced slimy matrix, known as extracellular polymeric substances (EPSs), which mainly comprise polysaccharides, extracellular DNAs (eDNAs), lipids, and proteins (Fig. 1*A*) (5–7).

Biofilms in bacterial species differ in their EPS composition, the production of which is orchestrated by various genes. Biofilms can increase the necessity of certain antibiotics up to 1000-fold to be effective against microorganisms and provide a protective niche to microorganisms against the host's immune defense (8–12). Biofilm-producing microorganisms exhibit altered physiology such as stunted growth, changed phenotype, overexpression of various resistance determinants including efflux pumps, and alterations in membrane and biofilm matrix composition (13, 14). The situation becomes more complex and severe due to the presence of persisters, a small subpopulation of cells that can tolerate exceedingly high doses of antibiotics (15). Biofilms are omnipresent and can be found in human body, natural, and man-made environments. A large number of both gram-positive and gram-negative bacteria are known to be associated with biofilm-mediated infections (16). Reports suggest that biofilm-producing microorganisms are involved in nearly 80% of chronic bacterial infections (1, 17, 18). A few well-known biofilm-associated bacterial infections are urinary tract infections, lung infections, cystic fibrosis (CF), and chronic tonsillitis. Medical implants and devices such as pacemakers, catheters, prosthetics, contact lenses, dentures, etc., are also highly susceptible to biofilm formation (Fig. 1*B*) (6, 19). Like bacteria, fungi also tend to form biofilms and flourish in aggregated communities enclosed in an extracellular matrix (20, 21). Over 65% of human fungal infections involve biofilm (21). Among fungi-related biofilm-mediated infections, *Candida* species are the ubiquitous etiologic agents. However, other yeasts and filamentous fungi like *Cryptococcus*, *Saccharomyces*, *Pneumocystis*, *Aspergillus*, *Trichosporon*, *Blastoschizomyces*, *Malassezia*, *Coccidioides*, etc. are known to infect individuals with indwelling medical devices such as catheters, pacemakers, and implants (22–25). Introduction of invasive medical implants into the patient's body forms an appropriate condition for fungi to adhere and form biofilms. Fungi are eukaryotic with complex cellular systems, which add to the difficulty in diagnosing and treating biofilm-mediated fungal infections. The situation is further aggravated by amplified cell density of sessile populations with altered physiological states and the expression of a specific array of genes (26). A recent study found that preformed biofilms were unaffected by high concentrations of most antifungal agents. For example, even newly adherent cells can grow, proliferate, and form biofilms in the presence of high concentrations of fluconazole (a common antifungal medicine that is used to prevent and cure fungal and

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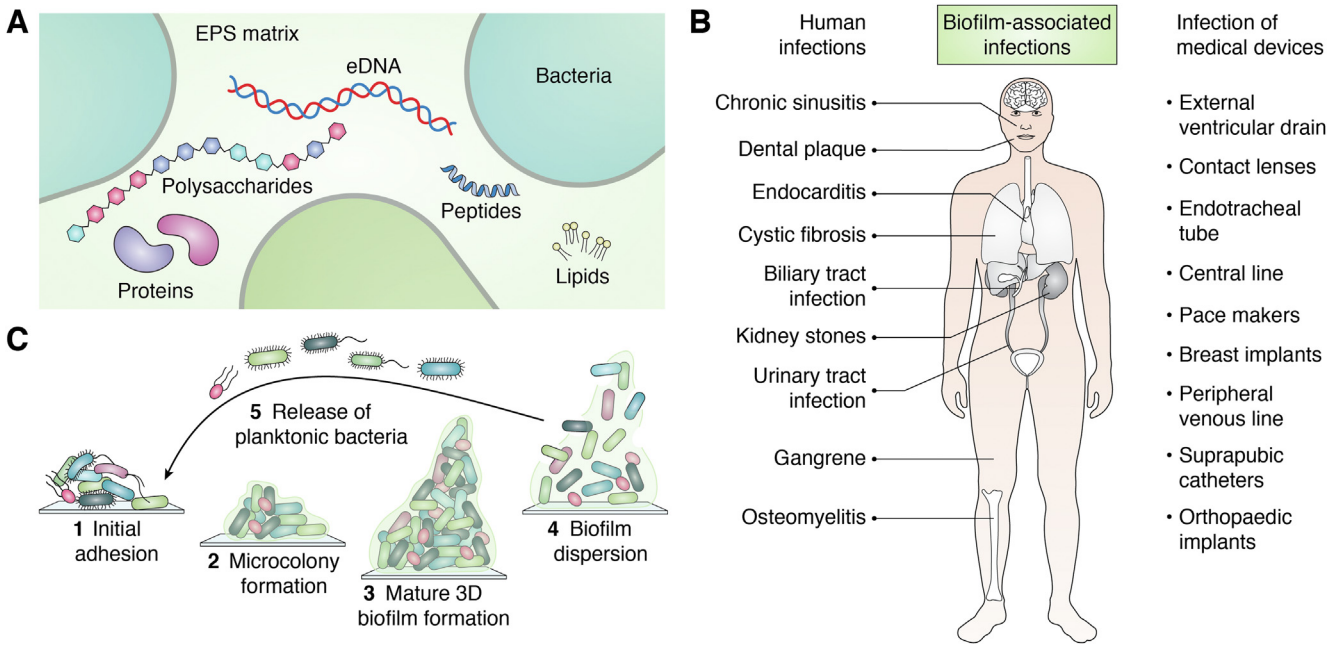

Figure 1. Biofilms and their impact on human health. *A*, the major components of the EPS matrix of biofilm. *B*, biofilm-associated infections in humans. *C*, the life cycle of microbial biofilm. EPS, extracellular polymeric substance.

yeast infections) (27). As a consequence, biofilm-mediated fungal infections are associated with high mortality rates.

Biofilms can be monomicrobial and polymicrobial, consisting of various bacterial strains, fungi, algae, etc., which results in a highly complex structure (5). Biofilm formation occurs in five stages: (1) migration and adhesion of cells on a suitable substratum; (2) microcolony formation by EPS secretion and cell aggregation; (3) multiple layer formation over the microbial colonies and their maturation; (4) attaining maximum cell growth and cell density; and (5) release of the mature and healthy cells to nearby sites for spreading the infection further (Fig. 1*C*) (28–31).

Since the first report of biofilms in the seventeenth century (32), research on biofilm formation by microorganisms has steadily increased as their contribution to pathogenicity is increasingly recognized. The current strategies to treat biofilms include both inhibition of biofilm formation and eradication of preformed biofilms. Biofilm formation can be inhibited by preventing the initial attachment of cells to surfaces or by arresting their maturation. Initial adherence of cells can be blocked either by modifying the attachment surface or preconditioning it with antimicrobials, which have been widely explored (33–35). In contrast, eradication of the preformed biofilms is more challenging as the recalcitrant polymer matrix must be degraded, leading the biofilm to disperse. Strategies that have been developed against biofilm-associated infection include administering antimicrobial, inhibition of quorum sensing (QS), and photodynamic therapy (Figs. 2 and 3). However, the major limitation of the existing methods is their poor biocompatibility. The quest for biocompatible antibiofilm agents has spurred interest in utilizing enzymes as potential therapeutic agents to treat biofilm-associated infections.

In this review, we briefly summarize the utility and limitations of the existing methods to combat biofilms; more details on these approaches can be found in various recent review articles (5, 9, 28, 36–38). Our main focus is on enzymatic biofilm dispersion, which has garnered substantial interest in recent years. We discuss an emerging enzyme-based strategy targeting one of the major components of the EPS matrix, the polysaccharides, for efficient dispersion of biofilms under physiological conditions.

Existing methods to combat biofilms

Antimicrobial coating/surface modification

Minimizing the initial microbial adherence to a substratum is a promising strategy to impede biofilm development. Adhesion depends on features such as surface roughness, charge, topography, stiffness, and environmental factors such as hydrodynamics (39). Disruption of the initial adhesion can

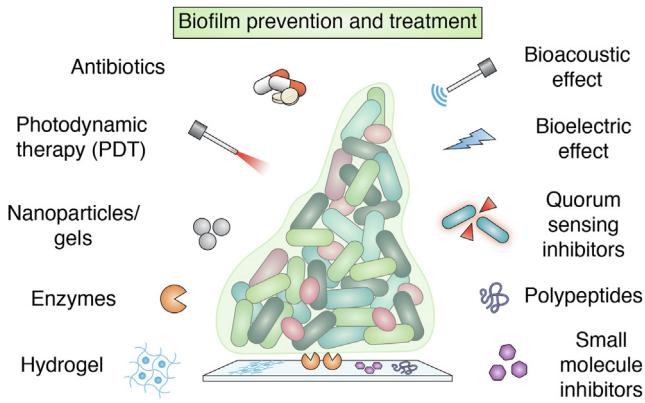

Figure 2. Methods to prevent and treat biofilms.

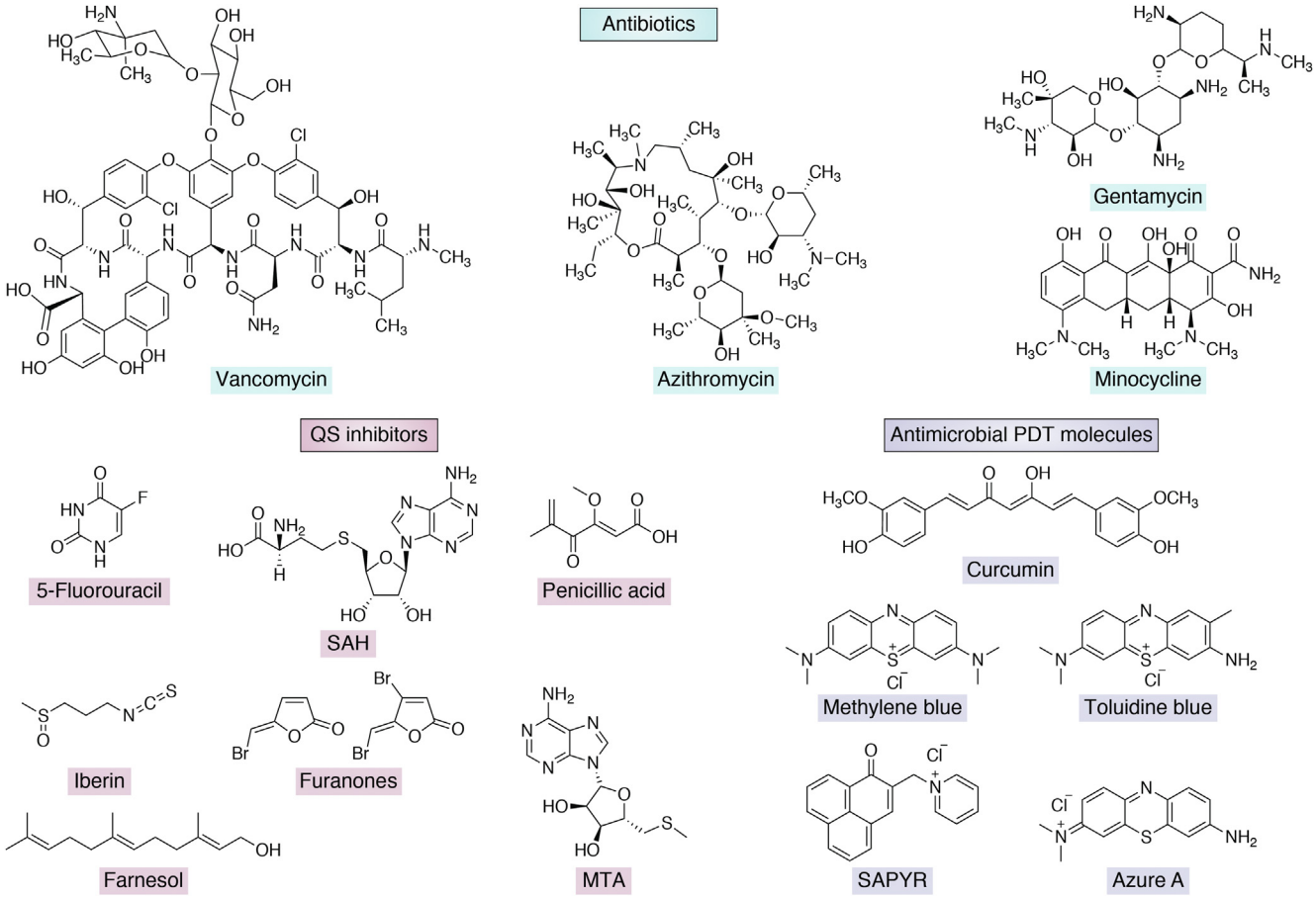

Figure 3. Examples of small molecules used for the treatments of biofilm-associated infections.

be achieved through surface modification employing antimicrobial coatings, which is a widely explored method against biofilms (40). For instance, coating albumin on polyethylene/polypropylene surface of medical devices significantly reduces bacterial adhesion (41). However, albumin coating requires additional chemical modification using functionalized cyclodextrins to facilitate the adsorption of albumin (42). Modification of intubations and prostheses made of silicones grafted with C1 and C8 alkyl side chains exhibited ~92% reduction in biofilm formation by *Candida albicans in vitro* (43). Similarly, coating the surface with cationic peptides Histatin5 and its synthetic variants led to a 93% reduction in biofilm formation by *C. albicans in vitro* (44, 45).

Nanoparticles were also found to be effective in reducing the surface adhesion of bacteria (46). Metal nanoparticles possess a positive charge that facilitates their electrostatic attraction to the negatively charged bacterial membrane and, therefore, can perturb the bacterial cells prone to adhere to the metal surface. Reports suggest that silver nanoparticles disrupt the bacterial cell membrane, trigger reactive oxygen species (ROS) generation, and thereby denature proteins, damage DNA, and affect the respiratory enzymes (47). Silver nanoparticle-coated medical implants such as urinary catheters and silver-loaded hydrogels are currently in use. Likewise, selenium, titanium, copper oxide, iron oxide, molybdenum, and curcumin nanoparticles/quantum particles have been explored (48–52). Although this strategy looks promising, it suffers from a lack of longevity in the application. Additionally, applying the metallic coating often requires elevated temperatures that may damage the nonmetallic implant materials (53).

Antimicrobial peptides (AMPs) have also been employed as surface coating agents against biofilm formation. AMPs exhibit broad spectrum antibacterial activities against both the gram-positive and gram-negative bacteria. AMPs target the functionality and stability of membrane and can kill microbes in the planktonic state, sessile state, and even in dormancy by pore formation (54, 55). Prominently, melimine has shown a broad spectrum activity targeting methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, fungi, protozoa, etc. (56) Various studies have demonstrated successful covalent immobilization of AMPs on medical implants and materials such as glass, titanium oxide, resin beads, and silicone surfaces. Nevertheless, the high production costs of AMPs and poor optimization of their properties have limited their clinical applications and commercialization (57).

Recent studies have shown that implants coated with biodegradable polymers and hydrogels could prevent the initial stages of biofilm formation. PEG and hyaluronan-based hydrogels are widely explored, which efficiently encapsulate

and systematically release antibiotics (58, 59). Controlled release of antibiotics to the implant site helps to minimize side effects. Even though antimicrobial agents play a significant role as a preventive measure by reducing the frequency with which implants need to be replaced, there remain challenges associated with the stability and longevity of coatings, toxicity elicited by them, and integration of surrounding indigenous tissues with the implant (60). Antimicrobial coatings can unpredictably promote biofilm formation due to the deposition of initial dead microorganisms killed by the coating (61). Further, the slow release of antibiotics can lead to the development of bacterial resistance (62).

QS inhibition

Another approach to inhibiting biofilm formation involves targeting the bacterial communication system known as QS that regulates a wide variety of bacterial physiology such as motility, virulence, symbiosis, competence, conjugation, sporulation, antibiotic production, as well as biofilm formation (63). After attaining the threshold cell density, bacteria produce *N*-acyl homoserine lactones (AHLs) and furanones (among gram-negative isolates), or autoinducer peptides (among gram-positive isolates), or autoinducer-2 (among some gram-positive and some gram-negative isolates), which help in modulating specific gene expression for the EPS production and expression of virulence factors (64, 65). AHLs are the primary QS molecules in gram-negative bacteria, which are produced by cognate AHL synthases. When the bacterial population increases and AHL molecules exceed their threshold concentration, specific receptors belonging to the class of DNA-binding transcription factors sense it and alter gene expression (66). Autoinducer peptides are signaling molecules released by membrane transporters. As their concentration rises, they bind to the corresponding histidine kinases, which phosphorylate downstream response regulators and ultimately change the expression of target genes (67). Although QS is not a prerequisite for bacterial growth, its quenching can be an excellent antivirulence strategy (68) and therefore help in inhibiting biofilm formation. Various enzymes, peptide-based agents, and small molecules have been explored extensively as QS inhibitors (69, 70). Specific lactonases and acylases were shown to degrade AHLs (71, 72). Analogs of the precursors of AHLs such as 5′-methylthioadenosine, *S*-adenosyl-homocysteine (Fig. 3), butyryl-SAM, and sinefungin have been employed against *P. aeruginosa* (73, 74). Further, the transition state analogs of 5′-methylthioadenosine have been shown to interrupt the QS of *Vibrio cholerae* and *Escherichia coli* (75). Investigators have also explored QS signaling antagonizing analogs such as *N*-octanoyl cyclopentylamides, *N*-nonanoyl cyclopentylamides, and *N*-decanoyl cyclopentylamides that exhibited anti-QS activity. Interestingly, *N*-decanoyl cyclopentylamides was also reported to exhibit strong antibiofilm activity (76). However, only a few of these quorum-quenching and antagonizing molecules have reached clinical trials owing to their ineffectiveness against the preformed, mature biofilms (77, 78). Azithromycin is used as a QS inhibitor for treating pulmonary transplanted and CF patients in clinical trials (79–81). *In vitro*, it disrupts the QS in *P. aeruginosa* strains; however, *in vivo* it was not effective (82). Natural QS inhibitors such as garlic extracts were also ineffective in clinical trials on patients (83). 5-Fluorouracil (5-FU), which is used as an anticancer drug, has been shown to prevent the expression of QS-regulated virulence factors in *P. aeruginosa*; therefore, its use as a coating agent for catheters was suggested in the clinical trials (84). 5-FU inhibits thymidylate synthetase, thereby inhibiting DNA replication. Studies suggest that 5-FU has various side effects on patients, and the molecule can affect the cardiovascular system (85). However, recently, Sedlmayer *et al*. have reported anti-QS activity of 5-FU against methicillin-resistant *S. aureus* in the murine model (86).

Bioelectric effect

Bacterial cells are sensitive to electric fields; in fact, electric fields have been used for making competent cells. In combination with antibiotics, electrical methods such as applying DC voltages, low AC currents, and pulsed electric fields have proved synergistic against biofilms produced by *P. aeruginosa* and *S. aureus in vitro* (87). Electric field applied in conjunction with QS inhibitors inhibited *E. coli* biofilm growth and showed propitious synergetic effects with gentamycin (88). These methods were even found effective in preventing planktonic bacteria from initiating biofilm formation. In spite of showing promising results in *in vitro* studies (77, 89), the bioelectric effect is minimally tested in animals and calls for further *in vivo* experiments before any conclusions on its utility can be made.

Bioacoustic effect

Ultrasonication can improve in the permeability of the biofilm to antibiotics (90–92), thereby reducing the amount of antibiotics required to inhibit bacterial growth. Ultrasonication combined with antibiotics substantially reduced biofilms formation by *E. coli*, *Staphylococcus epidermidis*, and *P. aeruginosa* in catheters (11, 77). The acoustic ultrasonic wave acts as a repulsive force by interfering with the attachment of planktonic bacteria to the surface. Most of the *in vitro* studies were performed in a sonication bath with the biofilm formed on implants. *In vivo* studies were carried out by administering antibiotics along with the ultrasound treatment using a small portable transmitter (93). The major challenge for *in vivo* studies is limited application time, which results in inefficient therapy, especially with indwelling medical devices.

Antimicrobial photodynamic therapy

Antimicrobial photodynamic therapy (PDT) is a noninvasive therapeutic approach that employs photosensitizers combined with near-infrared lasers and oxygen to suppress biofilm-associated infections (94, 95). PDT involves two steps: first a photosensitizer is administered and then light irradiation generates photoexcited molecules that react with ambient

oxygen to generate ROS. The ROS react with the proteins and eDNA present in the EPS and thereby disrupt the biofilm. The photosensitizer molecules are chosen based on their ability to bind to microbial cells, as efficient attachment is important for antibiofilm activity—a selection of these molecules is depicted in Figure 3. It is possible that the photosensitizer molecules are sequestered by EPS or partially penetrate through the biofilm or bind to microbial cells. *In vitro*, antimicrobial PDT has shown promising efficacy in biofilm eradication. Further, PDT has proven efficient in *in vivo* models such as implants inserted in mice, biofilm-associated wounds, burn injuries, etc. Clinical studies suggest that PDT can treat oral biofilm formed on teeth with negligible side effects (96). However, over-accumulated photosensitizer on untargeted areas can cause burns, redness, pain, and swelling. Therefore, this technique is mainly applied where the topical or local administration of photosensitizer is possible, rather than by intravenous or oral systemic administration (95).

Enzymatic degradation of polysaccharides—an emergent approach of biofilm dispersion

Success in the war against drug-resistant microbes invariably requires effective dispersion of biofilms under physiological conditions. The EPS matrix contributes up to ∼90% of the total biofilm. The dissolution of the EPS matrix of mature biofilm to gain access to the indwellers (microbes that live inside the biofilm) is an emerging area of biofilm research. Polysaccharides are the major component of the EPS matrix, which helps microbes to adhere to a variety of different surfaces. Further, polysaccharides provide immense strength and inertness to the biofilm and thereby protect the microbial population from desiccation, filter out antimicrobials, act as nutrient reservoirs, and facilitate formation of a suitable structured microenvironment for microbes to persist (97). For instance, the primary polysaccharide associated with *S. aureus* and *S. epidermidis* biofilms is polysaccharide intercellular adhesion, a partially de-*N*-acetylated homopolymer composed of β-1,6-linked 2-amino-2-deoxy-D-glucopyranosyl residues (98). Bacteria can also modulate the degree of *N*-acetylation and *O*-succinylation depending upon their requirements (99). Polysaccharide intercellular adhesion is referred to as poly *N*-acetyl glucosamine (PNAG) in gram-negative bacteria, which lacks the machinery for *O*-succinylation (100, 101). Likewise, *P. aeruginosa* biofilms contain three different polysaccharides, namely Pel, Psl, and alginate. The chemical structure and composition of Pel were unclear for a long time. Recent studies have suggested that Pel is a cationic exopolysaccharide with partially de-*N*-acetylated 1,4-linked *N*-acetylgalactosamine (102–104). The nonmucoid and mucoid isolates of *P. aeruginosa* secrete Psl, which is a polymer of pentasaccharide repeating units consisting of D-mannose, D-glucose, and L-rhamnose (103, 105). Mucoid *P. aeruginosa* isolates produce Psl and alginate that is a random linear polymer of acetylated 1,4-linked β-D-mannuronic acid and its C5 epimer α-L-guluronic acid (106, 107). Pel and Psl are found in the sputum of individuals with CF, and these exopolysaccharides facilitate aggregation of *P. aeruginosa* in CF airways, which helps in biofilm formation that results in chronic infection (103). Further, capsular polysaccharides contain repeating monosaccharides and are extensively hydrated, mainly involved in the biofilms of the members of Enterobacteriaceae such as *Klebsiella pneumoniae* and *E. coli*. Besides, in their biofilms, both cellulose and colanic acid or M antigen are present (108). The biofilms of *Streptococcus mutans* are primarily composed of the neutral homopolymer of β-D-fructans with irregular and extensive branching, commonly termed levans (97). A list of the well-characterized polysaccharides associated with the biofilms produced by various pathogenic bacteria and fungi is shown in Table 1 (97, 102, 109–126). Further, the structures of a few of these polysaccharides are depicted in Figure 4.

Degradation of the polysaccharides could weaken the biofilm substantially and render the sessile microbial population easily accessible to antimicrobial, thereby ensuring better clearance of the microbes from the infected areas. Interestingly, indwellers of biofilm are known to produce saccharolytic enzymes to initiate biofilm dispersal events in response to nutrient deprivation (127). It has been found that the gram-negative bacterium *Actinobacillus actinomycetemcomitans* produces a glycoside hydrolase (GH) enzyme, Dispersin B, for the dispersal of self-produced mature biofilm (128). Dispersin

Table 1
Various pathogenic microbes and the polysaccharides present in their biofilms

Pathogenic microbes	Major polysaccharides in the biofilm
Salmonella enterica	Cellulose (β-1,4)
Pseudomonas aeruginosa	Alginate (β-1,4) and (α-1,4), Pel (1,4), Psl (α-1,2) (β-1,3), (α -1,3)
Streptococcus mutans	Glucans (α-1,6), levan (β-D-fructans)
Staphylococcus epidermidis	(β-1,6), 2-deoxy-2-amino-D-glucopyranosyl residues, PNAG (β-1,6)[a]
Staphylococcus aureus	PNAG (β-1,6)[a]
Escherichia coli (Uro-pathogen)	PNAG (β-1,6), Cellulose (β-1,4), colanic acid (α-1,4 & β-1,3)
Klebsiella pneumoniae	PNAG (β-1,6)
Enterobacter spp.	Colanic acid (α-1,4 & β-1,3), *N*-acetylheparosan
Proteus mirabilis	*N*-acetyl-D-glucosamine (β-1,4), *N*-acetyl-L-fucosamine (α-1,3), D-glucuronic acid (α-1,3)
Bacterioides fragilis	{,3) α-D-AATGal*p*(1,4)[β-D-Gal*f*(1,3)] α-D-Gal*p*NAc(1,3) β-D-Gal*p*(1, }[b]
Serratia marcescens	Stewartan, Emulsan, polysaccharide B, capsular polysaccharide
Aspergillus fumigatus	Galactosaminogalactan (partially deacetylated heteropolymer of α -1,4-linked galactose and N-acetyl galactosamine)
Candida albicans	α-1,2 branched and α-1,6 mannans

[a] In gram-positive bacteria PNAG is known as polysaccharide intercellular adhesion (PIA) that has *O*-succinylated groups.
[b] AATGal: Acetamido-amino-2,4,6-trideoxygalactose. The galactopyranosyl residue has a pyruvate substituent. D-Gal*p*: D-galactopyranose; D-Gal*p*NAc: *N*-acetyl-D-galactopyranosamine; D-Gal*f*: D-galactofuranose.

B is a β-*N*-acetylglucosaminidase enzyme that catalyzes cleavage of the β-(1,6)-linked PNAG, which is a major component of the exopolysaccharides of biofilms produced by *E. coli, Pseudomonas fluorescens*, *Actinobacillus pleuropneumoniae* (gram-negative bacteria), as well as *S. epidermidis* and *S. aureus* (gram-positive bacteria). Binding of the polysaccharide substrate to the active site of Dispersin B is assisted by a set of conserved residues: one Glu, three Trp, and one Tyr (129). The site of cleavage of the glycosidic bond (endo- or exo-) of the polysaccharide by Dispersin B depends on the nature of the substrate (129–131). The active site architecture of the enzyme (Protein Data Bank [PDB] ID: 1YHT) (132) displays a conserved Asp-Glu dyad, where the Glu residue performs the general acid-base catalysis to hydrolyze the glycosidic bond through an oxazolinium intermediate (Fig. 5*A*). The Asp residue assists the N-acetyl group for nucleophilic attack. The ability of Dispersin B to disrupt biofilms has stimulated several attempts to utilize a similar strategy for effective dispersion of biofilms.

Sourjik *et al.* have engineered an *E. coli* strain by introducing Dispersin B into the microorganism, which could disrupt the biofilm containing PNAG (133). In an alternative strategy, Collin *et al.* have developed a T7 phage that induces a gene encoding an EPS degrading enzyme to the phage genome. The engineered phage led to efficient dispersal of *E. coli* biofilm and bacterial cell lysis (134). However, the specificity of bacteriophage targeting the host systems and release of toxins upon bacterial lysis makes this approach unpromising for clinical applications. Further, quick clearance by phagocytes in the human body makes phage therapy challenging and limited to the laboratory scale disruption of biofilms. *Staphylococcal* species and *P. aeruginosa* are the common bacterial strains found in most biofilm-related infections (77). Recently, Baker *et al.* have shown that nanomolar concentrations of GHs (PelA and PslG) present in the periplasm of *P. aeruginosa* can disrupt biofilm formed by the microorganism *in vitro* (135, 136). They have also shown that treatment of *Aspergillus fumigatus* (an opportunistic fungal pathogen) biofilms with GHs Sph3 or PelA significantly improved the activity of the antifungal agents such as posaconazole, amphotericin B, and caspofungin. Both Sph3 and PelA were found to be noncytotoxic. The intratracheal administration of Sph3 was well tolerated and led to a substantial reduction of fungal outgrowth within the lungs of a neutropenic mouse model (136). Asker *et al.* have shown that PslG can be uniformly immobilized to commercially available medical grade polyethylene, polyurethane, and polydimethylsiloxane catheter tubing. Moreover, the chemically conjugated PslG was found substantially reduce the formation of *P. aeruginosa* biofilm both *in vitro* and *in vivo* (137, 138). Rumbaugh *et al.* have investigated the effect of two commercially available GHs, α-amylase from *Bacillus subtilis* and cellulase from *Aspergillus niger*, on biofilms produced by *S. aureus* and *P. aeruginosa*. Their investigation suggests that the enzymes could disrupt the polymicrobial biofilm produced by the two microorganisms (139). Structural investigation of α-amylase from *B. subtilis* in the polysaccharide bound form

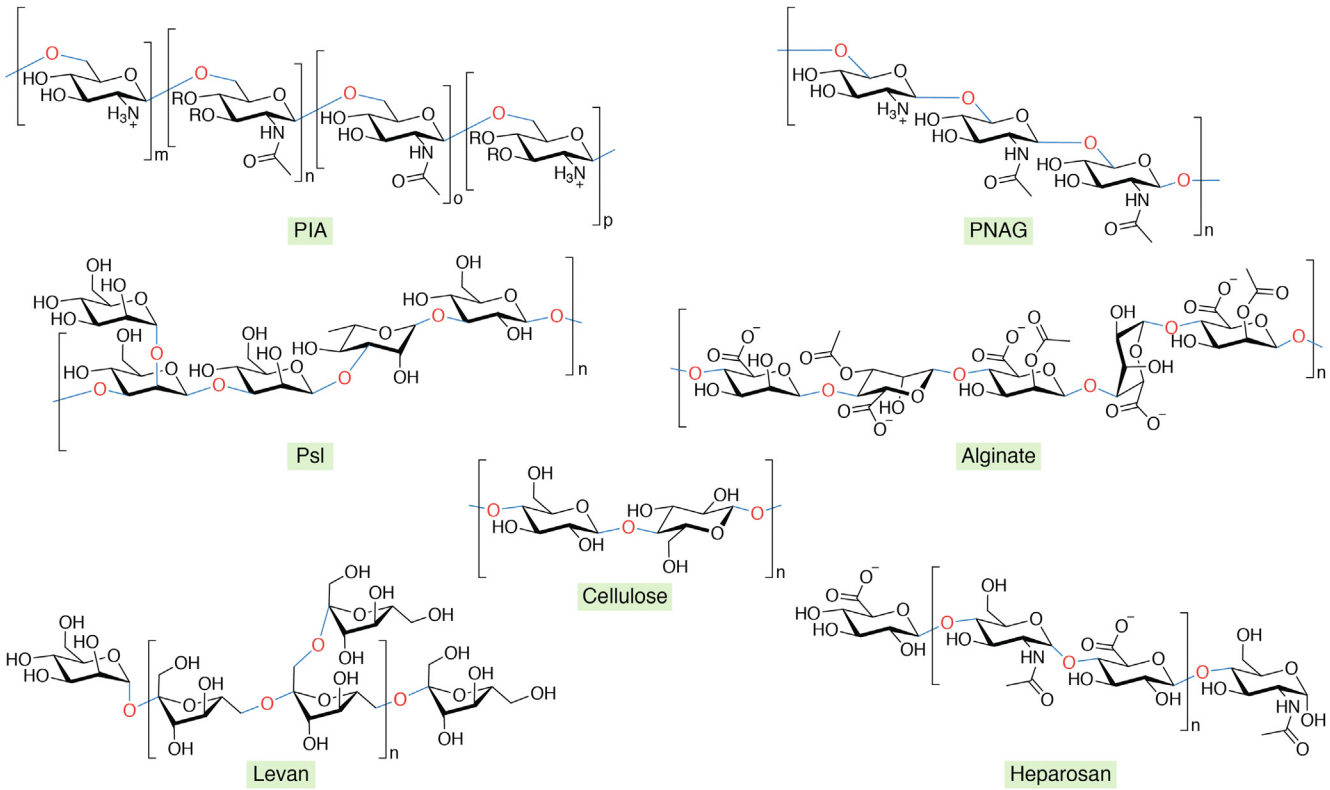

Figure 4. Structures of the polysaccharides found in the EPS matrix of biofilms produced by various pathogenic bacteria. *O*-succinylation of PIA is indicated by the R group. EPS, extracellular polymeric substance; PIA, polysaccharide intercellular adhesion.

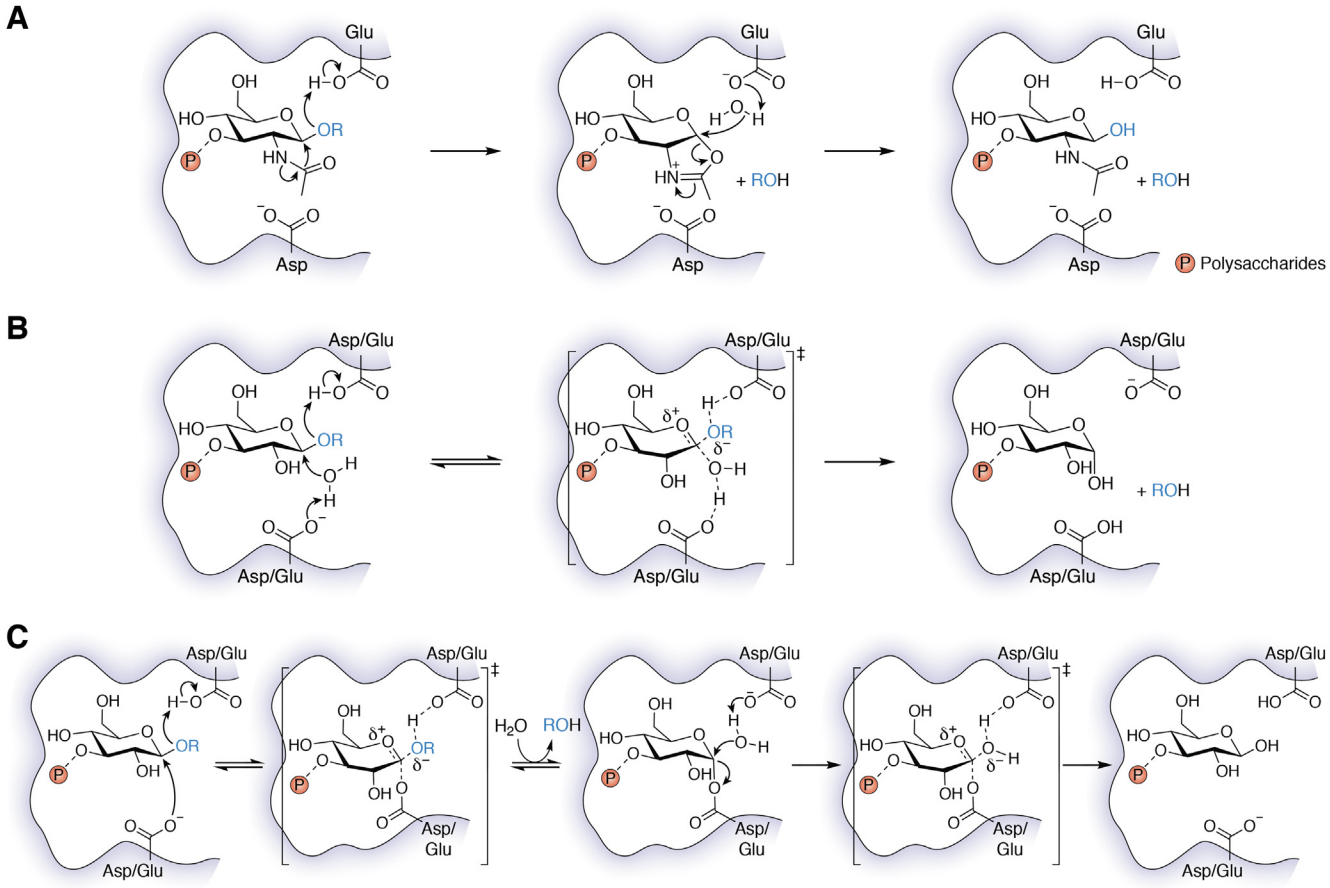

Figure 5. Plausible mechanisms of cleavage of glycosidic linkages of polysaccharides in biofilm by GH enzymes. *A*, the mechanism of Dispersin B, which involves oxazolinium intermediate during the catalysis. *B*, the inverting mechanism of GH, which follows a concerted pathway. *C*, the retaining mechanism of GH, which involves the formation of an enzyme-substrate covalent intermediate. In all cases, conserved Asp/Glu residues are involved in acid-base catalysis. The orange circle with P stands for polysaccharides. GH, glycoside hydrolase.

(PDB ID: 1BAG) demonstrates the involvement of three conserved active site acidic residues, two Asp and one Glu, forming a triangle around the cleavage site (140). While one conserved residue Glu and Asp serves as the general acid and base for catalysis, the other conserved Asp residue has a vital role in substrate binding. Similar to α-amylase, cellulase harbors two conserved Glu residues at the active site (PDB ID: 5I77) for catalysis (141).

Both the α-amylase and cellulase are well-known GHs that have been employed for various industrial applications. α-Amylase catalyzes cleavage of the α-(1,4) glycosidic bonds of starch, glycogen, and several other oligosaccharides (140). Cellulase catalyzes hydrolysis of the β-(1,4) glycosidic bond, which is one of the most commonly found linkages in the exopolysaccharides present in biofilms formed by various pathogenic strains such as *S. aureus*, *Salmonella enterica*, *P. aeruginosa*, *E. coli*, etc. (142) Besides, preclinical studies suggest that cellulase and α-amylase treatment can enhance antibiotic intervention to clear the infection in a murine surgical excision wound model (18). In another study, the Rumbaugh group found six GHs that could disrupt monomicrobial and polymicrobial biofilms produced by *S. aureus* and *P. aeruginosa* (143). These pioneer studies demonstrate that GHs could be generally effective for biofilm dispersal. Although the mechanisms of these enzymes have not been investigated in the context of biofilm dispersion, it can be presumed that the enzymes follow the general acid-base catalysis involving conserved Asp/Glu residues to cleave the glycosidic linkages of the polysaccharides present in the biofilm (Fig. 5, *B* and *C*) (144–146).

Jee *et al.* have demonstrated that the combination of α-amylase and protease (bovine pancreas type-I) significantly inhibits the biofilm growth of *E. coli*, *S. aureus*, and methicillin-resistant *S. aureus* (147). Polysaccharide lyase (PL) is another class of enzymes that cleaves uronic acid–containing polysaccharides using a β-elimination mechanism (148) and thereby disrupt the EPS matrix of the biofilm. Again, cleavage enhances the effect of antibiotics and phagocytosis in the eradication of biofilm-associated infections (149). Recently, a PL, isolated from *K. pneumoniae* bacteriophage (SH-KP152226), was shown to degrade biofilm produced by multidrug-resistant *K. pneumoniae* (150). Alginate lyases from the PL class of enzymes have also been explored for the disruption of *P. aeruginosa* biofilms (151). Although various reports indicate the combinatorial therapy of antibiotics with alginate lyase effectively reduces the minimum inhibitory concentration of multiple antibiotics (149), Lamppa *et al.* have shown that alginate lyase–mediated biofilm dispersion and the

synergy of antibiotic are not coupled with the enzyme activity (152). Although alginate lyases manifest biofilm-dispersive properties, there is controversy regarding the antibiofilm effect of this enzyme. DNase I is another class of enzyme known to degrade biofilms. Disruption of eDNA of the biofilm matrix increases the vulnerability of the biofilm to antibiotics as the negative charge of eDNA is known to sequester cationic antibiotics (153). DNase I, sold as Dornase alfa, is clinically prescribed as a mucolytic agent for CF patients. It acts on the DNA of sputum, helping to reduce its viscosity and allowing it to be cleared (154). Nevertheless, the efficacy of the treatment in the CF patients is more related to compliance, as the enzyme needs to be nebulized and inhaled over 1 h, which is uncomfortable for the patients. *In vitro* studies suggest that DNase I treatment might be beneficial in preventing the establishment of chronic *P. aeruginosa* infection of the CF lung by inhibiting biofilm formation (155). Recently Pirlar *et al.* have demonstrated that the synergistic action of DNase I and trypsin helped to disperse the polymicrobial biofilm of *S. aureus* and *P. aeruginosa* in the wound-like medium, which resulted in a 2.5-fold reduction in the minimum concentration of antibiotic needed for biofilm eradication (156).

Nevertheless, investigations suggest that enzymes, particularly the polysaccharide degrading enzymes, might hold a key to the effective dispersion of biofilms produced by many pathogenic microbes. The chemical structures and the nature of the glycosidic linkages of various polysaccharides present in the biofilms of a large number of microbes have been explored (Table 1). Therefore, selectively targeting biofilms using GH enzymes might be feasible. A list of these enzymes used for biofilm dispersion is shown in Table 2 (135, 139, 157–179).

The pilot studies of biofilm dispersion (*vide supra*) by various GH enzymes serve as the proof of concept that GH enzymes have substantial potential for biofilm dispersion. The ability of GH enzymes to disperse mature biofilms both *in vitro* and *in vivo* makes them unique from other physicochemical methods (180). Many GHs have been studied from thermophilic fungi due to interest in these enzymes for recalcitrant biomass degradation at high temperatures for biofuel production. However, such enzymes function poorly under physiological conditions, restricting their ability to effectively degrade the EPS matrix's polysaccharides in biofilms. Therefore, mesophilic GH enzymes, preferably from mammalian microbiome, which would catalyze biofilm dispersion under physiological conditions with minimal immunogenic effect, are urgently needed to combat biofilm-related infections. The CAZy database holds information on thousands of carbohydrate-active enzymes from various microorganisms, and the recent surge in the metagenomic analysis of biomass-degrading microbes has tremendously increased the entries of novel GHs in the CAZy database (181). Such analysis also includes the discovery of numerous saccharolytic enzymes from ruminant animals such as cows and goats that have cellulose-rich diets (182–184). This observation suggests that some of the most active mesophilic cellulolytic enzymes from the mammalian microbiome may serve as potential candidates for effective dispersion of biofilms. Such enzymes can play a pivotal role in degrading biofilm by which the microbes become planktonic, which then can be killed by the low dose of drugs. EPS-degrading enzymes thus may be of potential therapeutic value in treating microbial infections. These enzymes may also be useful to dislodge microbial colonies from the implants, which otherwise can make the implant nonfunctional. It is important to note that enzymes are typically noncytotoxic to sessile microbes; instead, they will disperse the biofilm by disrupting the EPS matrix and provide easy access of the drugs or the host immune system to the indwellers (Fig. 6*A*). Therefore, broad spectrum antimicrobial activity could be achieved by combining these enzymes with antimicrobial drugs. Such combinatorial therapy has shown

Table 2
Reported glycoside hydrolases for biofilm dispersion

Glycoside hydrolase	Targeting glycosidic linkage	Monosaccharide composition of the polysaccharides
Dispersin B	GlcNAc -(β-1,6)- GlcNAc	*N*-acetyl-D-glucosamine
α-Amylase	Glc-(α-1,4)-Glc	D-glucose
Cellulase	Glc-(β-1,4)-Glc	D-glucose
PslG	Man*p*-(β-1,3)- Man*p*[a]	D-mannose, D-glucose and L-rhamnose
PelA	(1,4)[b]	Partially de-*N*-acetylated *N*-acetylgalactosamine
β-Mannosidase	Man-(β-1,4)-Man	D-mannose
α-Mannosidase	Man-(α-1,3)-Man	D-mannose
PgaB	GlcN-(β-1,6)-GlcN	D-glucosamine
Ega3	GalN-(α-1,4)-GalN	D-galactosamine
Sph3	GalNAc-(α-1,4)-GalNAc	*N*-acetyl-D-galactosamine
CcsZ	Glc-(β-1,4)-Glc	D-glucose
PssZ	ManN-(β-1,4)-ManN	D-mannosamine

[a] The targeting glycosidic linkage of PslG is predicted.
[b] PelA enzyme works on Pel, a linear cationic polysaccharide composed of partially de-N-acetylated 1,4 linked N-acetylgalactosamine.

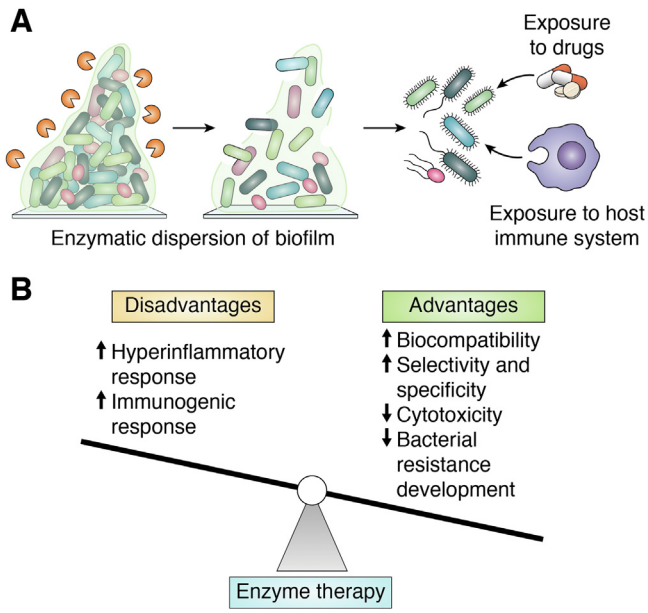

Figure 6. Enzymatic dispersion of biofilms: advantages and disadvantages. *A*, enzymatic dispersion of biofilm can lead to facile access of the drugs and the host immune system to the indwellers. *B*, advantages and disadvantages of enzyme therapy. The disadvantages of enzyme therapy can be dealt with established methods. The *black arrows* indicate the likely increase or decrease of various responses and factors related to the enzyme therapy.

promising results in the efficient treatment of chronic wound infections (27, 139). Nonetheless, a few challenges associated with the microbial enzyme therapy need to be addressed: these include the immunogenicity, short *in vivo* half-life of the enzymes, cost, and applicability of enzymes in the clinic. Immunogenicity toward foreign proteins is unavoidable in the host systems, which may require further protein modification such as PEGylation (185, 186), development of chimeric protein system (187, 188), or chemical modifications (189) to nullify the off-target effects. PEG is a nontoxic and amphipathic polymer, widely used for modulating enzyme activity and pharmacokinetics to protect from immunoreactivity, immunogenicity, and *in vivo* degradation of the enzymes (190). In addition, enzyme encapsulation and modification with bioconjugates such as antibodies, peptides, hormones, vitamins, and DNA are under study to enhance the *in vivo* stability and reduce immunogenicity and clearance (191, 192). Cost, however, may not be a major factor for utilizing enzymes for biofilm dispersion. Heterologous overexpression and purification of enzymes using microorganisms such as *E. coli* and yeast has revolutionized the field of protein biochemistry. The process gives an opportunity for strain manipulation, overexpression and faster protein production, easy scale up, recovery, and purification (193). In conjunction with the inexpensive gene synthesis, the approach has become an economically viable option for protein production.

Enzyme therapy on mixed species biofilm is still a challenge due to the substrate-specific nature of most enzymes. Thus, efficient dispersal of biofilm requires multienzyme formulations capable of degrading polysaccharides, eDNA, and QS molecules present in the EPS matrix (194, 195). Moreover, the enzymatic unmasking of biofilm can cause huge microbial exposure to the nearby tissues, which may elicit hyperinflammatory responses. These responses may vary by organism and the site of infection; however, the combination of enzymes with antimicrobials can successfully overcome these challenges (139). A great advantage of implementing enzyme therapy for biofilm eradication is that it is very unlikely that the biofilm-forming pathogens will rapidly develop resistance to the enzymes since the targets of the enzymes are the polysaccharides of the EPS matrix and not the microorganisms. It is noteworthy that bacteria can modify the molecular mass of polysaccharides and can introduce nonsugar residues by expressing enzymes such as pyruvyl or acetyl transferases (196). Also, they can change the comonomer composition by employing epimerases (197). Further, they can alter elastomeric properties by covalently crosslinking the polymers. Nevertheless, GHs mainly target the glycosidic linkages, so these modifications will not affect the inherent efficacy of the enzymes directly but may restrict the access of the enzyme to the linkages. However, such limitations may be overcome through protein engineering. Nevertheless, the high selectivity, specificity, and biocompatibility of the enzymes make them superior to other biofilm therapeutics (Fig. 6*B*).

Enzyme therapies have been successfully employed in various fields, such as the medical and food industries. For example, collagenase is used for dispersion of ulcers and burns and is also used for the neurosurgical treatment of Dupuytren's contracture (198, 199). Lipases from microbial sources are used to treat gastrointestinal disturbance and dyspepsia (200), and bovine pancreatic enzymes are used for the treatment of pancreatic cancer, chronic pancreatitis, and CF (201, 202). Even though the preclinical studies of enzymatic biofilm dispersion are promising, (203) only limited demonstrations of the safety and efficacy of the enzymes in animal models are currently available, and more *in vivo* studies are needed to bring them to clinical trials.

Conclusion and perspectives

With recent advancements in our understanding of the mechanisms of microbial infection, clinical solutions have been employed to treat patients, yet biofilm-associated infections loom as a grave concern to human health. Biofilm-associated infections develop slowly, and the symptoms show up at a much later phase of infection. Facile disruption of biofilms without adverse side effects is highly desired for effective antimicrobial treatments of biofilm-associated infections. Although current approaches such as antimicrobial coating, bioelectric effect, bioacoustic effect, and photodynamic therapy have shown promise *in vitro*, only in a very few cases biocompatibility has been demonstrated *in vivo*. A major limitation of the monoantimicrobial and combinatorial antimicrobial drug therapy is the need for a much higher dose at the biofilm-associated infection site. The dose required is often significantly more than the allowed therapeutic dose, leading to severe hepatotoxicity and nephrotoxicity. The challenges associated with the existing methods have motivated scientists to innovate more effective solutions to biofilms. In this review, we have highlighted one such approach —the enzymatic dispersion of biofilms, which is an emerging avenue of research. In our assessment, GH enzymes, preferably from the mammalian microbiome, which target polysaccharides of the EPS matrix, hold great potential for developing first-in-kind enzyme therapy for dispersion of mature biofilm under physiological conditions. The specificity and lower chances of rejection by the human body may give enzymes an upper hand over the existing physicochemical strategies.

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Abbreviations—The abbreviations used are: 5-FU, 5-fluorouracil; AHL, *N*-acyl homoserine lactone; AMP, antimicrobial peptide; CF, cystic fibrosis; eDNA, extracellular DNA; EPS, extracellular polymeric substance; GH, glycoside hydrolase; PDB, Protein Data Bank; PDT, photodynamic therapy; PNAG, poly *N*-acetyl glucosamine; PL, polysaccharide lyase; QS, quorum sensing; ROS, reactive oxygen species.

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