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Review

Pseudomonas aeruginosa Biofilm Formation and Its Control

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Abstract: Microbes are hardly seen as planktonic species and are most commonly found as biofilm communities in cases of chronic infections. Biofilms are regarded as a biological condition, where a large group of microorganisms gets adhered to a biotic or abiotic surface. In this context, *Pseudomonas aeruginosa*, a Gram-negative nosocomial pathogen is the main causative organism responsible for life-threatening and persistent infections in individuals affected with cystic fibrosis and other lung ailments. The bacteria can form a strong biofilm structure when it adheres to a surface suitable for the development of a biofilm matrix. These bacterial biofilms pose higher natural resistance to conventional antibiotic therapy due to their multiple tolerance mechanisms. This prevailing condition has led to an increasing rate of treatment failures associated with *P. aeruginosa* biofilm infections. A better understanding of the effect of a diverse group of antibiotics on established biofilms would be necessary to avoid inappropriate treatment strategies. Hence, the search for other alternative strategies as effective biofilm treatment options has become a growing area of research. The current review aims to give an overview of the mechanisms governing biofilm formation and the different strategies employed so far in the control of biofilm infections caused by *P. aeruginosa*. Moreover, this review can also help researchers to search for new antibiofilm agents to tackle the effect of biofilm infections that are currently imprudent to conventional antibiotics.

Keywords: *Pseudomonas aeruginosa*; biofilm; quorum sensing; antibiotics; resistance; medicinal plants; enzymes; *in silico* screening

Citation: Vetrivel, A.; Ramasamy, M.; Vetrivel, P.; Natchimuthu, S.; Arunachalam, S.; Kim, G.-S.; Murugesan, R. *Pseudomonas aeruginosa* Biofilm Formation and Its Control. *Biologics* **2021**, *1*, 312–336. https://doi.org/10.3390/biologics1030019

Academic Editor: Leodevico L. Ilag

Received: 18 August 2021
Accepted: 11 October 2021
Published: 15 October 2021

1. Introduction

Naturally, microorganisms exist either as free-floating cells or enclosed within an architectural structure known as biofilms [1]. One of the preferred growth states for bacteria is a biofilm, which exists in more than 90% of bacteria [2]. In such an environmental niche, the bacterial communities are regulated by various biological processes and use advanced genotypic events to promote different molecular mechanisms and phenotypes that are necessary for survival in the new environment during pathogenesis and antibiotic treatment [3]. Thus, a biofilm is regarded as a group of microorganisms encased within a self-secreted polymeric extracellular substances matrix attached to a surface irreversibly and difficult to be detached by a tender rinse [4,5]. Biofilms form on a huge range of surfaces that includes living tissues, hotels, industrial places, labs, wastewater channels, bathrooms, indwelling medical devices, and are frequently found on hard surfaces immersed in or exposed to an aqueous solution [6]. Nearly 99.9% of all microbes can develop biofilms on both biotic and abiotic surfaces [7].

Biofilms are heterogenous with 15% of cells, usually in microcolonies, and 85% of polymeric extracellular substances. The composition of the biofilm matrix varies among different species, but in general contains proteins, polysaccharides, and nucleic acids [8].

Structural support and protection for bacteria in biofilms are rendered by the extracellular matrix [9]. The matrix is also involved in various other processes such as adherence to surfaces, cell-to-cell communication, quorum sensing (QS), tolerance, etc. [1].

Biofilm matrix development and bacterial growth are dependent on factors, namely, availability of nutrients and hydrodynamic conditions [10]. The cooperative interactions among species lead to various development states, structures, and functions of biofilm organization [11]. Biofilms are considered polymicrobial and hence there is a huge rivalry for nutrients and space. The cohabitation of numerous microbes on a surface promotes cooperative behaviors such as metabolic cooperation, horizontal gene transfer, and other synergies, thereby leading to an increased potential of microorganisms to survive and exhibit resistance to antimicrobial agents [12,13].

The presence of biofilms on man-made surfaces imparts its significance in connection to pathogenicity, whereas biofilm formation in undesirable places may lead to medical and industrial complications as they show resistance to cellular immunity in the host, antimicrobial, and biocide treatments [1]. This concept of biofilm was first discovered by Anton Van Leeuwenhoek in 1684 when he was observing the surface of a tooth using a primitive microscope [14]. A biofilm could not be removed with ease adopting a standard clinical procedure and could be detached only by complete elimination of the infected implant, which increases the trauma rate of the patients and treatment cost [15].

Resistance to antibiotics is approximately 1000 fold more in attached bacteria than planktonic cells because of an increase in mutation rates, upregulation of efflux pumps, decrease in metabolic activity, and other physical reasons [16]. The resistance mechanism is unique to biofilm-encapsulated bacteria as the biofilm phenotype provides a protective advantage [17].

Biofilms hold a significant role in healthcare-associated infections (HAI), in particular those connected to the implant of medical devices, namely urinary catheters, orthopedic implants, and intravascular catheters. Annually, as reported by the European Center for Disease Prevention and Control (2008), around 4,100,000 patients acquire HAI in European hospitals, and the number of deaths due to these infections are estimated to be around 37,000 [18]. Approximately, there are 200,000 cases of bloodstream infections in the United States every year due to implants of central venous catheters [19].

According to recent reports, it is estimated that 449,334 patients are affected per year in US hospitals due to catheter-associated urinary tract infections (UTIs) [20]. Among various biofilm-associated infections, UTIs are the most common bacterial infections affecting humans and serve as a public health issue [21]. Nevertheless, in 2017, bloodstream infections associated with biofilms were ranked as the 12th leading cause for death with an overall mortality rate between 15–30% [22].

At present, approximately 80% of all microbial infections are of biofilm origin, out of which, 60–70% are nosocomial infections caused by biofilms on implanted medical devices [23]. In a clinical environment, nosocomial infections account for merely 65% of hospital-acquired infections [24,25]. Even after more than 70 years since the first report on biofilms [26], still there is a need in various areas such as biomedical and environmental fields related to the problems encountered by biofilms [27,28].

In such a case, the most studied organism related to QS and biofilms is the Gram-negative bacterium *P. aeruginosa* as it is one of the most virulent opportunistic pathogens, which leads to a variety of acute infections and continues to possess a high rate of mortality and antibiotic failure [29]. According to literature reviewed recently, *P. aeruginosa* was found to be the fourth most frequently found pathogen, contributing to around eight percent of chronic wound infections, and the seventh leading pathogen, contributing to around two to six percent of bloodstream infections. Further, epidemiological studies have proved that infections of *P. aeruginosa* could significantly increase the rate of mortality, morbidity, need for surgical intervention, chronic care, and overall cost of treatment [30]. Hence, focusing on the potent treatment strategies to prevent *P. aeruginosa* associated biofilm infections is the present area of concern.

2. Formation of Biofilm

Biofilms may be defined as "Microbial communities consisting of various bacterial cells living in close association by encasing itself in an extracellular matrix made up of polymeric substances (EPS), adhered to a substratum or each other and exhibit an altered phenotype" [14,31]. The growth of bacteria within biofilms is a naturally occurring phenomenon in which the whole of the microbe could be lively attached to an infection site. The ability of the bacteria to colonize the environment and to mature as a biofilm on a surface is considered as one of the survival strategies for biofilm-forming microorganisms [32].

In general, microorganisms are found as organized groups that grow on diverse surfaces to constitute a distinct growth phase compared to free-swimming planktonic cells [33]. Formation of biofilm is a complex and cyclic phenomenon that involves transportation, diffusion, chemical reaction, ecological mechanisms, and is controlled by mechanisms that include bulk transport, adhesion, quorum sensing, detachment, death of cells, and dispersal [34,35]. Thus, biofilms are regarded as structural architectural organizations of microorganisms that evolve constantly to get adapted to their surroundings [36].

Bacterial biofilms could be well established in a few hours [37]. The formation of biofilm comprises of four main stages: (a) Initial attachment of planktonic bacteria to a surface through physical forces and interaction occurs between bacteria and surface of attachment; (b) Adherent cells gets attached to the surface irreversibly and encase themselves in extracellular polymeric substances matrix resulting in aggregation of cells; (c) Maturation of biofilm by microcolony formation to form a three-dimensional architecture of completely matured biofilm; (d) Release of microcolonies of cells from the matured biofilm to colonize new attachment site for spreading its infection [38].

Synthesis of extracellular matrix holds a crucial role in the biofilm development as it incorporates all the elements that make up 90% of the total organic matter o found in the matrix material, the most important structural feature of bacterial biofilms [39]. The components of the matrix include nucleic acids, lipids, polysaccharides, and proteins [40].

The functions of the matrix elements are to deceive nutrients, give structural support, and provide protection against natural resistance and antibiotic therapies in the host [41]. EPS holds all the cells of biofilm in the near vicinity to enable intercellular interactions (QS) and facilitate the genetic material exchange by gene transfer method [42]. It is reported that extracellular DNA (eDNA) is necessary for pathogens to adhere and for its cell-to-cell coherence at the early stage of biofilm development [43].

The characteristic features of a matured biofilm in general are, [34]

1. Adherence to each other
2. Adherence to either solid/liquid, solid/air, liquid/liquid, or liquid/air interfaces
3. Attachment to surfaces
4. Decreased antimicrobial susceptibility
5. Decreased host defense systems
6. Existence of one or more microbial species
7. Three-dimensional structure

At present, biofilms are well known to possess a biological role and pose a significant issue in medicine as they are responsible for a lot of healthcare-related diseases. According to the National Institute of Health (NIH), it is identified that biofilms formed by bacteria account for around 65% of infectious diseases caused by microbes and chronic infections by about 80% [38]. The most common biofilm-associated infections caused in humans are chronic sinusitis, wound infection, osteomyelitis, prosthetic joint infection, prosthetic valve endocarditis, infections of cystic fibrosis patients, ventilator-related pneumonia, intravascular catheter infection, and breast implant infections [44].

Biofilms cause infections by colonizing on inert surfaces, on dead tissues, on living tissues, and more commonly by dwelling on implanted devices like contact lenses, orthopedic implants, urinary catheters, peritoneal dialysis catheters, central venous catheters, prosthetic joints, pacemakers, mechanical heart valves, voice prostheses, implantable electronic devices, and other orthopedic and dental implants made up of composites and

ceramics [15,38,45]. Some of the other non-device-related infections are chronic infections, periodontitis, and osteomyelitis [46]. Microbial adhesion in implanted medical devices may be made of single or multiple types of microbial species depending on the device and its duration of action but are more severe and can cause life-threatening complications [38].

The matured biofilms could be detected by several biofilm detection methods that can be categorized into four divisions: physical, chemical, microscopical, and biological techniques [47]. Some of the notable techniques include congo red agar, tube culture, microtiter plate assay, and in particular, the biofilm architecture could be studied elaborately by examining it using confocal laser scanning microscopy, optical sectioning, three-dimensional imaging, and scanning electron microscopy [31,34]. These biofilm techniques also possess certain properties such as in situ monitoring, real-time monitoring, and online monitoring, which can categorize and qualify the biofilms formed, and are representative, reproducible, accurate, and automatic [47]. The results obtained from these techniques are the 2D distribution of bacteria in the biofilm, total cell counts, the 3D structure of biofilm, microbial activity, and identification of different components of biofilms [48].

A typical type of potent biofilm causing infectious microorganisms are *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Pseudomonas pseudomallei*, *Haemophilus influenza*, *Escherichia coli*, *Candida albicans*, *Streptococcus pyogenes*, *Streptococcus pneumonia*, other *Streptococcus species*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* [15,45,49]. Among these, *S. aureus* and *S. epidermidis* contribute to about 87% of bloodstream infections, 50–70% of catheter infections and, 40–50% of prosthetic heart valve infections [50]. Though *S. aureus* and coagulase-negative staphylococci are associated with the majority of implantable device-related infections, *P. aeruginosa* can readily adapt itself to harsh environments and antibiotics instantly, thus making it a suitable in vitro model for studying biofilm formation [51].

3. *Pseudomonas aeruginosa* Biofilms

P. aeruginosa is a virulent rod-shaped, Gram-negative bacterium belonging to the group of Pseudomonadaceae and found extensively inhabiting the water, plants, soil, and animals, which hardly cause infections in healthy individuals, but can easily cause infections in immune-compromised individuals [52]. For more than a decade, *P. aeruginosa* is among the 'top 10' common hospital 'superbugs' because of its widespread antimicrobial-resistant strains that cause life-threatening complications [53]. It is the most commonly isolated species from chronic wounds and is considered a potent biofilm producer since they act as a barrier in wound healing and exhibits high resistance to antimicrobial therapy [54,55].

According to US National Healthcare Safety (2007), *P. aeruginosa* was ranked to be the sixth most commonly occurring organism responsible for nosocomial infections, second most common pathogen responsible for ventilator related pneumonia, and seventh major causative pathogen of catheter-linked bloodstream infections accounting for high death rate in individuals with AIDS, cystic fibrosis, and burn wounds [56–58].

P. aeruginosa causes infections with the aid of several cell-based virulence factors such as pili, lectins, alginate, lipopolysaccharide, and secreted virulence factors, namely, pyocyanin, cytotoxin, proteases, hemolysins, siderophores, exotoxin A, exoenzyme U, exoenzyme S, etc. respectively [59].

The mechanism intricated in the development of biofilm by *P. aeruginosa* is initially a free-floating bacterium gets reversibly adhered to a conditioned surface, then the adherent bacteria are irreversibly attached by surface adhesins followed by the formation of an extracellular matrix to produce a completely matured biofilm. Finally, dispersion of bacteria occurs from the matrix to colonize other surfaces (Figure 1).

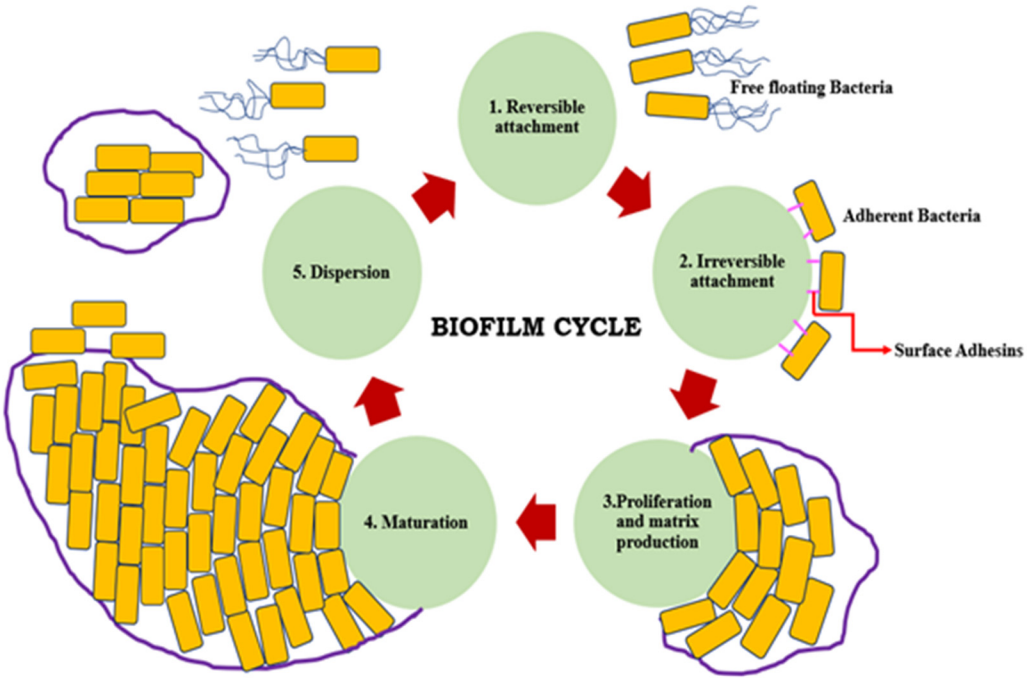

Figure 1. Cyclic process of biofilm formation in *P. aeruginosa*.

The stability of *P. aeruginosa* biofilm structure is determined by various polysaccharides, which include alginate, pel, and psl [60,61]. Alginate is an unbranched polymer chain consisting of D-mannuronic acid and L-glucuronic acid. This polymer is essential for the protection and stability of the biofilm structure. Alginate also contributes to the preservation of contents of the matrix such as nutrients and water [62]. Pel polysaccharide is a matrix material enriched with glucose, but with its composition still unknown and psl is a pentasaccharide composed of repeating residues of D-mannose, L-rhamnose, and D-glucose. Both these polysaccharides are implicated in the initial biofilm development stages by serving as a primary structure scaffold [63–65]. Another crucial element of *P. aeruginosa* biofilm is eDNA, which is regarded as a nutrient source for embedded bacteria and plays a key role in cell-to-cell interconnection [8,66].

Synthesis of alginate, pel, and psl polysaccharides is regulated by bis-(3-5)-cyclic dimeric guanosine monophosphate (c-di-GMP), an intercellular ubiquitous second messenger that is widespread in bacteria [67]. Higher concentrations of c-di-GMP promote alginate and pel polysaccharides production and lower concentrations of c-di-GMP enhance the motility of bacteria, but the exact mechanism that regulates the polymerization of these polysaccharides precursors are still unknown [68].

Each polysaccharide is encoded on unique sites of the genome in which alginate is coded by a 12 gene operon, pel by a 7 gene operon, and psl by a 12 gene operon. The functions of different genes encoded by alginate, pel, and psl polysaccharides are presented in Table 1 [65,69,70].

Table 1. Functions of alginate, pel, and psl polysaccharide encoded genes.

Polysaccharide	Gene	Function	References
Alginate	*AlgD*	Sugar nucleotide production	[71]
	Alg8	Subunit polymerization	[72]
	Alg44	c-di-GMP binding	[73]
	AlgK	Outer membrane protein/secretion	[74]
	AlgE	Outer membrane protein/secretion	[74]
	AlgG	Epimerase/modification	[75]
	AlgX	Epimerase/modification	[76]
	AlgL	Hydrolase/lyase	[77]
	AlgI	O-Acetylation	[78]
	AlgJ	O-Acetylation	[76]
	AlgF	O-Acetylation	[78]
	AlgA	Sugar nucleotide production	[79]
Pel	*PelA*	Hydrolase/lyase	[80]
	PelB	Outer membrane protein/secretion	[81]
	PelC	Outer membrane protein/secretion	[82]
	PelD	c-di-GMP binding	[83]
	PelE	Subunit polymerization	[84]
	PelF	Glycosyl transferase	[85]
	PelG	Inner membrane protein	[86]
Psl	*PslA*	Subunit polymerization	[87]
	PslB	Sugar nucleotide production	[79]
	PslC	Glycosyl transferase	[72]
	PslD	Outer membrane protein/secretion	[88]
	PslE	Wzz/Wzc like protein	[89]
	PslF	Glycosyl transferase	[90]
	PslG	Hydrolase/lyase	[91]
	PslH	Glycosyl transferase	[90]
	PslI	Glycosyl transferase	[90]
	PslJ	Inner membrane protein	[86]
	PslK	Inner membrane protein	[86]
	PslL	Inner membrane protein	[86]

4. Role of Quorum Sensing (QS) in Biofilm Formation

An intercellular signaling system known as QS imparts a major part in the formation of biofilms by regulating gene expression using small molecules called autoinducers [92]. The development and structural integrity of the biofilm is merely dependent upon QS [93]. QS is a cell-to-cell interconnection mechanism that prevents cell density from reaching a threshold level to control its population density [94].

A level at which the autoinducers reach a threshold concentration at a specific cell density is referred to as "quorum level". At this level, autoinducers bind to their respective receptors to increase or decrease the activity of several genes responsible for maintaining the size of biofilm and coordinating phenotypic virulence [40,95]. Thus, the viability of the biofilm community is always dependent upon quorum sensing or quorum diffusions [93].

Generally, QS networks in Gram-negative bacteria and Gram-positive bacteria are modulated by signaling molecules such as N-acyl homoserine lactones and oligopeptides, respectively. Another signaling molecule, namely, autoinducer-2 (AI-2) regulates QS in both types of bacteria [96]. Gram-negative bacteria, *P. aeruginosa* access Acyl Homoserine Lactones (AHLs) as its signaling molecule for regulating its QS networks. AHL molecules consist of a fatty acyl chain connected by an amide bond to lactonized homoserine. Different AHL molecules are synthesized by various bacterial species or the same bacterial species may synthesize different AHLs. Variations in composition of acyl chains contribute to various physiological and biochemical functions of the bacterial species [97].

A wide range of regulatory proteins involved in the QS mechanism of Gram-negative bacteria have been identified, among them, LuxR-type protein is the widely studied model since most of the members of this protein are AHL-responsive transcriptional activators [98]. LuxR-type proteins have two domains, namely, an N-terminus acyl-HSL-binding site and a C-terminus DNA binding site. The binding of acyl-HSL to the N-terminus domain promotes configurational changes that enable multimerization and DNA binding for transcriptional activation of the associated promoters [99,100].

The advantage of QS lies not only in controlling population density, but also in spreading beneficial mutations to colonies of biofilms, which induce accessibility to nutrients and tolerance to antibiotics [101]. Inadequacy in the regulation of QS networks alters the structure and architecture of biofilms [62].

As QS pose a trivial impact on most of the regulatory processes, interrupting this mechanism serves as a critical approach and target of interest to control biofilm-forming pathogens [102,103]. Many approaches have been so far reported to hinder QS such as blocking of signal receptors and signal transduction, enzymatic degradation of signaling molecules, preventing autoinducers synthesis, etc., which destroyed biofilms completely by the host immune system [104,105].

5. *Pseudomonas aeruginosa* Quorum Sensing System

Among the various QS systems studied in different species, the *P. aeruginosa* QS system is considered an important one because of its severe pathogenicity. *P. aeruginosa* QS system is controlled by various pathways and exhibits interrelated effects [106]. The importance of the QS system in *P. aeruginosa* biofilms was first reported in 1998 by Davis and his group [107]. Four types of QS systems have been so far studied in *P. aeruginosa*. They are *las*, *rhl*, *pqs*, and *integrated QS (IQS). IQS* was added to the *P. aeruginosa* QS system recently and hence its mechanism is not much exploited [108]. The *las* system is made up of LasI synthase that induce the synthesis of signaling molecule N-(3-oxododecanoyl)-L-homoserine lactones (3-oxo-C12-HSL), recognized by its LuxR-type receptor protein LasR to activate transcription of target genes. In the *rhl* system, the signaling molecule N-butanoyl-L-homoserine lactone (C4-HSL) synthesized by RhlI synthase is recognized by its signal receptor RhlR to induce regulation of target gene expression [109]. Among these two LuxR-type receptor proteins, LasR is activated the earliest and regulates the expression of RhlR [110]. Both *las* and *rhl* systems are not only involved in biofilm formation but also regulate various gene expressions necessary for virulence factors production [108].

LasR protein is made up of two independently folded domains, an N-terminus ligand binding region, and a C-terminus DNA binding region [111]. By comparing the wild-type strain of biofilm with that of mutant lasI biofilm strains, Davies et al. [107] reported the importance of the *las* system in the development and maturation of biofilms. The mechanism of LasR is, LasR stabilizes itself and undergoes dimerization on binding to a signaling molecule and the resulting LasR homodimer complex regulates the transcriptional activation of target genes [112]. It was reported by Gilbert et al. [113] that LasR binds to the psl operon in the promoter region and regulates psl expression.

On the other hand, RhlR binds to C4-HSL or an alternative signaling molecule synthesized by PqsE, a thioesterase intricated in alkyl quinolone synthesis to activate genes necessary for the production of virulence-associated QS factors and formation of biofilms [114].

C4-HSL does not stabilize RhlR [115] like the way LasR is stabilized since RhlR does not bind C4-HSL tightly, as evidenced by Boursier et al. [116]. The *rhl* system of *P. aeruginosa* is involved in biofilm formation by modulating the synthesis of Pel polysaccharides [62].

The third QS system *pqs* synthesizes signaling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) that recognizes its cognate receptor PqsR for regulating eDNA release during biofilm formation [117,118]. Apart from this, the *pqs* system also regulates other metabolic processes in *P. aeruginosa* such as the secretion of elastase, rhamnolipid, the formation of membrane, and so on [119,120]. Many researchers suggested *pqs* as important for virulence and it is increasingly seen in patients with cystic fibrosis affected by *P. aeruginosa* infections [121–124].

Recently discovered QS system *IQS* produces autoinducer molecule 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde, which is sensed by IqsR and modulated by *las* and PhoB, a phosphate stress response regulator. *IQS* controls the production of PQS, C4-HSL, and virulence factors, namely, elastase, rhamnolipids, and pyocyanin [119,125]. The interconnected QS network in *P. aeruginosa* is depicted in Figure 2.

P. aeruginosa QS system possess hierarchical relationships among them with *las* system in the top position since it regulates other QS systems and *rhl* at the lowest position as it is regulated by other QS systems to activate QS-related virulence factors. PQS activates *rhl* and is regulated by *las* and *IQS*. *IQS* controls *pqs* and *rhl* systems and is activated by *las* [119,126]. Though the *P. aeruginosa* QS system is an interlinked network, each system can be controlled by several environmental factors including phosphate stress [127], starvation [128], low oxygen [109], low iron [129], and host-derived factors [119,125].

In addition to the above, sigma factors like RpoS and RpoN, global regulators of transcription such as AlgQ, MvaT, DksA, and VfR, and two important homologs of LuxR, namely, QscR and VqsR, are also involved in the regulation of the QS signaling circuit [109]. Among these, the LuxR homolog QscR is an orphan QS control repressor receptor protein that utilizes LasR's signaling molecule since it does not have a synthase enzyme. QscR serves as a negative regulator of the QS system by repressing both *las* and *rhl* systems, thus holding a pivotal position in the *P. aeruginosa* QS system [130,131]. A two-component system, GacS/GacA system is considered as a super-regulator of the QS network and regulates virulence-associated factors production and formation of biofilms [132,133].

Interlink between QS and biofilm formation has been described indirectly by regulation of twitching and swarming motilities, rhamnolipid, and lectin production [62]. Swarming motility, an organized form of surface translocation is useful in the early stages of biofilm development and is regulated by the *rhl* system [134,135]. Twitching motility, a flagella independent way of translocation necessary for microcolony formation is controlled by *rhl* on Fe-limited minimal medium [136,137].

Rhamnolipids production, which is regulated by *rhl*, is involved in various aspects of biofilm formation such as the formation of microcolonies, maintaining the open channel structure, facilitating mushroom-shaped 3-D structures, and aiding cell dispersion [118,138,139]. The galactophilic lectins LecA and LecB also support biofilm development and are under the control of the *rhl* system [140,141].

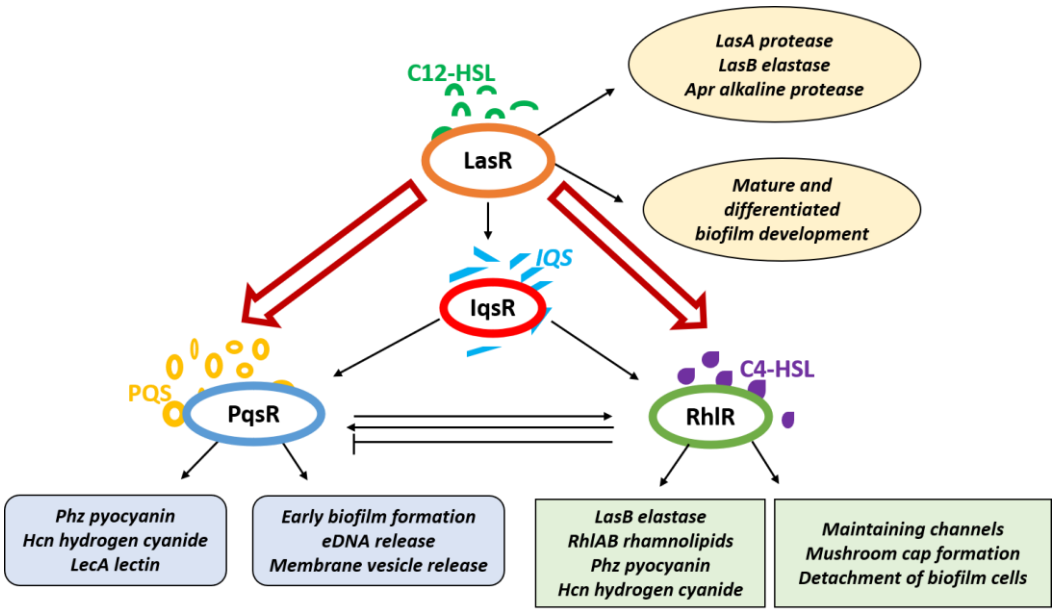

Figure 2. Quorum sensing mechanism of *P. aeruginosa*.

6. *Pseudomonas aeruginosa* Biofilm Challenge to Antimicrobial Agents

Planktonic cells are at greater risk to the effect of antibiotics and are sufficiently sensitive to antimicrobial agents, whereas bacteria within a biofilm structure are not susceptible to host immune system and antimicrobials as they exhibit a high tolerance and resistance to antimicrobial agents. Most of the resistance mechanisms of microorganisms are transferable and devoid of the target's interaction with antibiotics [36,142].

The biofilm structure of *P. aeruginosa* exhibit a greater extent of antibiotic resistance due to various reasons such as moderate or deficient penetration of antibiotics [143], the altered chemical environment within the biofilm [144], and cell differentiation in a biofilm [145]. All these mechanisms occur due to the multicellular nature of biofilms, thereby leading to antibiotic resistance of biofilm structure and failure in treatment strategies [146,147].

It has become a great challenge to give treatment to patients with infections of *P. aeruginosa* as they exhibit high-level resistance to most of the available antibiotics in use [148]. Recently, World Health Organization (WHO) has mentioned *P. aeruginosa* as a life-threatening species for which new antibiotics has to be developed to prevent its infections [149]. To date, empirical antibiotic therapy is used to treat cases of *P. aeruginosa* infections, but more use of antibiotics for therapy may develop multidrug-resistant strains of *P. aeruginosa* and can cause failure of empirical antibiotic therapy against this microbe [150–152].

P. aeruginosa possesses antibiotic resistance by various mechanisms, namely, intrinsic, acquired, and adaptive resistance mechanisms [153]. The intrinsic resistance includes decreased permeability to the outer membrane, efflux pumps expression, and synthesis of enzymes that inactivate antibiotics, whereas the acquired type of resistance includes mutational changes or horizontal transfer of genes responsible for resistance and the final adaptive resistance is implicated in biofilm formation in the lungs of infected patients that can act as a diffusion barrier to lower antibiotics from reaching the bacterial cells [154].

In addition to the above resistance mechanisms, multidrug-tolerant persister cells can form in the biofilms that withstand antibiotic attacks and cause prolonged periodic infections in individuals with cystic fibrosis [155]. Persisters are a bacterial subpopulation that possess a multidrug tolerance phenotype rather than genetic variations [156,157]. Mulcahy et al. [155] have evidenced high levels of persister cells in cystic fibrosis patients compared to wild-type strains of *P. aeruginosa*, suggesting them as highly antibiotic-resistant and to become multidrug-tolerant [158].

Most of the biofilm cells enter the stationary phase with time and persister cells are high in number at this phase. The main reason for decreased susceptibility to antibiotics is that 1% of the population in the stationary phase becomes tolerant [159,160].

The antibiotic-resistant state of biofilm cells are responsible for human infections by forming biofilms on medical implants such as heart and urinary catheters, heart valves replacements, and implants of joints [161]. They exhibit a serious threat to humans due to their pathogenicity and contribute to a majority of pathogenic infections [162,163]. Based on various observations, Sharma et al. [164] has stated, the multicellular developmental process of the biofilms could be considered for the opening of new targets and approaches to treat antibiotic-resistant microorganisms.

7. Strategies to Control *P. aeruginosa* Biofilm Infections

Increased rates of mortality and morbidity are seen in health care facilities among patients affected by *P. aeruginosa* infections due to failure in developing new antibiotics and its widespread resistance [165]. The development of new antibiofilm strategies could be effective to treat biofilm-related infections, thereby reducing their complications [166]. The following outlines the different alternative approaches established for fighting infections associated with *P. aeruginosa* biofilms.

7.1. Plants as a Natural Source of Antibiofilm Agents for P. aeruginosa Biofilms

For the past two decades, novel approaches in preventing QS and biofilm formation have been employed by natural products from plants that demonstrate chemo-protective and antimicrobial properties. It is already well known that natural products and herbal remedies have been used in practice by different human cultures for many years for therapy and to prevent the spread of infectious diseases [167]. The following table presents the various natural plant-based products as anti-biofilm agents for treating *P. aeruginosa* biofilm-associated infections (Table 2).

Table 2. Plant species screened to treat *P. aeruginosa* biofilms.

S. No	Plant Species	Plant Part	Extract	References
1	*Allium cepa*	Outer scales	Methanol	[168]
2	*Allium sativa*	Bulbs	Methanol	[168]
3	*Ananas comosus*	Fruit	Aqueous	[169]
4	*Centella asiatica*	Leaves	Ethanol	[170]
5	*Citrus sinensis*	Seeds	Methanol	[168]
6	*Coriandrum sativum*	Fruit	Methanol	[168]
7	*Couroupita guianensis*	Fruit	Chloroform	[171]
8	*Elettaria cardamomum*	Seeds	Methanol	[168]
9	*Euphorbia hirta* L.	Aerial parts	Methanol	[172]
10	Garlic	Bulbs	Toluene	[173]
11	*Hemidesmus indicus* (L.)	Root	Ethanol	[174]
12	*Holarrhena antidysenterica*	Bark	Ethanol	[174]
13	*Laurus nobilis*	Leaves	Methanol	[168]
14	*Mangifera indica* L.	Seed	Ethanol	[174]
15	*Manilkara zapota*	Fruit	Aqueous	[169]
16	*Mentha longifolia*	Aerial part	Methanol	[168]
17	*Musa paradiciaca*	Stem	Aqueous	[169]
18	*Ocimum sanctum*	Leaves	Aqueous	[169]
19	*Panax notoginseng*	Roots	Aqueous	[175]
20	*Psidium guajava*	Leaves	Methanol	[168]
21	*Psoralea corylifolia* L.	Seeds	Ethanol	[174]
22	*Senecio brasiliensis*	Stem bark	Ethanol/Aqueous	[176]
23	*Syzygium aromaticum*	Bud	Hexane, Chloroform, Methanol	[177]
24	*Terminalia catappa*	Leaves	Methanol	[178]
25	*Amphypterygium adstringens*	Stem bark	Hexane	[179]
26	*Sclerocarya birrea*	Stem bark	Methanol	[180]
27	*Ocimum basilica*	Whole plant	Aqueous	[181]
28	*Brassica oleracea*	Whole plant	Aqueous	[181]
29	*Zingiber officinale*	Whole plant	Aqueous	[181]
30	*Myristica cinnamomea*	Bark	Methanol	[182]
31	*Melicope lunu-ankenda*	Leaves	Hexane, Chloroform, Methanol	[183]
32	*Psidium guajava*	Leaves	Methanol	[168]

Table 2. *Cont.*

S. No	Plant Species	Plant Part	Extract	References
33	*Phyllanthus amarus*	Whole plant	Hexane, Chloroform, Methanol	[184]
34	*Capparis spinosa*	Dried fruit	Methanol	[185]
35	*Thymus* sp.	Whole plant	Aqueous	[181]
36	*Nymphaea tetragona*	Whole plant	Aqueous	[186]
37	*Terminalia bellirica*	Fruits	Methanol	[187]
38	*Terminalia chebula*	Fruits	Methanol	[187]
39	*Syzygium cumini*	Seeds	Methanol	[187]
40	*Sclerocarya birrea*	Bark	Methanol	[180]
41	*Punica granatum* L.	Pericarp	Ethanol	[174]
42	*Triumfetta welwitschii*	leaves	Dichloromethane: methanol	[188]
43	*Corchorus olitorius*	stem	Ethanol	[189]
44	*Phrynium capitatum*	Leaves	Ethanol	[190]
45	*Dryptes indica*	Leaves	Ethanol	[190]
46	*Plantain herb*	Whole plant	Ethanol	[191]
47	*Cinnamomum camphora*	Bark	Distilled water	[192]
48	*Centella asiatica*	Leaves	Ethanol	[170]
49	*Anogeissus acuminata*	Whole plant	Methanol	[193]
50	*Mallotus roxburghianus Muell*	Whole plant	Ethanol	[193]
51	*Camellia kissi wall.*	Leaves	Methanol	[194]
52	*Plectranthus tenuiflorus*	Leaves	Methanol	[195]
53	*Persicaria maculosa*	Aerial parts	Ethanol	[196]
54	*Bistorta officinalis*	Rhizome	Ethanol	[196]
55	*Syzygium legatii*	Leaves	Acetone	[197]
56	*Syzygium masukuense*	Leaves	Acetone	[197]
57	*Syzygium species A*	Leaves	Acetone	[197]
58	*Berginia ciliate*	rhizome with skin	Methanol	[198]
59	*Lavandulacoronopifolia*	aerial parts	Methanol: water	[199]
60	*Centella asiatica*	Leaves	Methanol	[200]
61	*Mentha spicata*	Leaves	Methanol	[200]
62	*Azadirachta indica*	Leaves	Methanol	[200]
63	*Psidium guajava*	Leaves	Methanol	[200]
64	*Syzygium aromaticum*	Whole part	Ethyl acetate	[200]
65	*Cinnamomum zeylanicum*	Whole part	Ethyl acetate	[200]

7.2. Enzymes against P. aeruginosa Biofilms

Another possible approach to control biofilms is the incorporation of enzymes to destroy polymers of the extracellular matrix and enable disruption of biofilms [166]. As biofilm matrix is a complex architecture, multi enzymatic formulations are needed to control biofilms effectively. Researchers have reported a variety of enzymes that can fight against *P. aeruginosa* biofilms [201].

A study conducted by Kovach et al. [202] reported that the effect of EPS-specific enzymes, namely, alginate lyase and DNase is greater on *P. aeruginosa* biofilms, whereas non-specific enzymes such as glycoside hydrolases, cellulases, and α-amylases did not significantly alter the biofilm mechanics in vitro. However, the mechanism of how these enzymes hinder the biofilm mechanism remains still unknown. In case of in vivo studies on a mouse model of wound infections, glycoside hydrolases were more productive than other specific enzymes since there might be a difference in the formation of biofilms developed in vivo and in vitro by genetically similar strains of bacteria.

Quorum quenching was initially described in 2000 with the invention of a quorum quenching enzyme from *Erwinia carotovora* that degrade AHL signals. Most of the identified quorum quenching enzymes namely phosphotriesterase-like lactonases (PLLs), lactonases, acylases, and oxidoreductases target AHLs [203–205]. Example of enzymes that can act as anti-biofilm agents for grafting of wounds and removes barriers that weaken wound healing, such as weakened tissues, bacterial biofilms, and scars, include bromelain derived debridase, collagenase, trypsin, fibrinolysin, lysozyme, streptokinase, and dispersin B [206–210]. Different classes of the enzyme known to control biofilms are shown in Table 3.

Table 3. Classes of enzymes used in controlling *P. aeruginosa* biofilms.

Class of Enzyme	Example	Target	References
Oxidoreductases	Glucose oxidase, Curvularia haloperoxidase	Directly or indirectly retarding bacterial growth by production of H_2O_2	[211,212]
Transferases	Transaminase	EPS matrix	[213]
Hydrolases	AiiA, α-amylase, Proteinase K	QS molecules, Exopolysaccharides, Exoproteins	[203,214,215]
Lyases	Alginate lyase	Exopolysaccharides	[216]

Acylase reduces *P. aeruginosa* ATCC 10145 and PAO1 growth to 60% by disrupting QS signaling [217,218]. A study by Vogel et al. [219] states immobilizing quorum quenching enzyme with quorum quenching properties such as acylase PvdQ over the polydimethylsiloxane silicone (PDMS) surface in a biosensor setup exhibited a 6-fold decrease of the auto-inducer 3-oxo-C12 compared to untreated material.

Researchers have stated enzymes namely lysozyme and proteinase K could inhibit the biofilm formation of many species of bacteria [220–222]. In connection to this, Eladawy et al. [223] reported lysozyme as an potent biofilm inhibitor as it reduces 19% biofilm formation at a physiological concentration of 30 µg/mL, whereas proteinase K exhibited a biphasic effect on *P. aeruginosa* biofilms at different concentrations. Another class of enzyme, BpiB09 oxidoreductases, were evidenced to reduce the motility of bacteria, biofilm formation, production of pyocyanin, and prevent the induction of N-(3-oxododecanoyl)-L-homoserine lactone in *P. aeruginosa* PA01 strains [224].

Dioxygenases destroy 2-heptyl-3-hydroxy-4 (1H) quinolone-based signaling molecules and have been shown to inhibit the quinolone signals in the *P. aeruginosa* QS system [225,226]. Banar et al. [227] studied the mechanism of β-glucosidase and lyticase enzymes on biofilms formed by various strains of *P. aeruginosa* and found that both enzymes degraded and altered the biofilm states. These enzymes also significantly reduced the colony-forming units and revealed no cytotoxicity when treated against cell lines of A-549 human lung carcinoma and A-431 human epidermoid carcinoma.

Another study by Daboor et al. [228] has revealed that alginate lyase (AlyP1400), a class of alginolytic enzyme purified from marine bacteria *Pseudoalteromonas* sp., reduced the *P. aeruginosa* biofilms after 24 h of incubation by 69% and could serve as a specific combinational therapeutic strategy when used along with conventional antibiotics. A lactonase group of quorum quenching enzyme, SsoPox-W263I reduces protease, elastase, and pyocyanin production among bacteriophage resistant strains or ten antibiotics and degrade acyl-homoserine lactones [229].

Snarr et al. [230] reported microbial glycoside hydrolases developed by recombinant technology may serve as a potent therapeutic agent with promising antibiofilm potential by destroying biofilms and inhibiting virulence. An enzyme-based endoscope cleaner was invented for clearing biofilms in medical devices by Stiefel et al. [231] using optimized selected base formulation of enzyme mixture consisting of polysaccharides, protease, and other enzymes, which removed about 90% of biofilms formed by *P. aeruginosa* in 96-well plate with >99% decrease of CFU and >90% decrease in extracellular polymeric substances.

A study on trypsin, α-mannosidase, and β-mannosidase enzymes effect on *P. aeruginosa* biofilm from wound infections caused due to burns by Banar et al. [232] proved only enzyme trypsin had no cytotoxic effect on cell lines of A-431 human epidermoid carcinoma and possessed features better than other enzymes, evidencing trypsin as a potential antibiofilm agent for treating burn wound infections of *P. aeruginosa*.

The only enzyme that is found to disrupt *P. aeruginosa* biofilms in clinical fields is Dornasealfa (deoxyribonuclease I), which promotes destruction by eDNA hydrolyzation within the extracellular matrix [233,234]. However, immature *P. aeruginosa* biofilms are more prone and sensitive when treated with deoxyribonuclease I than the mature biofilms [43,235]. Another therapeutic enzyme, glycoside hydrolase DspB (Dispersin B), hydrolyze the poly-b-1,6-N-acetyl-D-glucosamine (PNAG/PIA) exopolysaccharide found

in biofilms [206,207,236], but this PNAG is not found in *P. aeruginosa* biofilms [237–239]. Baker et al. [240] identified naturally derived glycoside hydrolases, namely, PelAh and PslGh that specifically target psl and pel polysaccharides, which can clear in vitro biofilms of clinical, environmental, and laboratory isolates at nanomolar concentrations.

A study by Kiran et al. [241] says lactonase treatment on *P. aeruginosa* biofilms significantly quenches all the major lactones synthesized by *P. aeruginosa* strains such as 3-oxo-C12-HSL and C4-HSL, which regulates virulence factor expression. In addition, extracellular hydrolases secreted by mucoid *P. aeruginosa* strains during biofilm growth EstA, LasB, and LipC can cause changes in the composition of EPS and alter the motility of cells as reported by Tielen et al. [242].

One of the main drawbacks of enzyme-mediated antibiofilm therapy is that it should be carried out in combination with antibiotic agents as it may become a preventive measure rather than a way of treatment. Another concern is their typical higher cost when compared to other costs of conventional chemical disinfectants and antimicrobial agents. But apart from this, many researchers have reported that biofilm matrix-degrading enzymes could be a potent antibiofilm agent to reduce the incidence of medical device infections [243].

7.3. In Silico Approach to Control P. aeruginosa Biofilms

Many studies have reported various chemical tools to exploit new knowledge to inhibit bacterial virulence by hindering the QS systems as a novel means to reduce *P. aeruginosa* infections efficiently, which makes it harder for the bacteria to develop drug resistance. Wang et al. [244] identified cladodionen, isolated from extracts of the marine fungal strain *Cladosporium* sp. Z148 as a novel QS inhibitor that showed effective binding conformation to LasR and PqsR compared to native ligands through molecular docking approach. He also reported that the QS-related mRNA gene expressions were down-regulated by cladodionen. A recent study by Sadiq et al. [245] reported sulfamerazine, a synthetic FDA-approved compound as an inhibitor of LasR by performing virtual screening and molecular docking by employing a pharmacophore hypothesis based screening and elucidating the stability of their binding conformation by a simulation study. A study by Baldelli et al. [246] suggested two antibiotic compounds, namely, nitrofurazone and erythromycin estolate as PqsE inhibitors by screening a library of FDA-approved drugs and found that these compounds reduce the expression of PqsE-dependent virulence and formation of biofilm in *P. aeruginosa* PAO1 model strain.

Abelyan et al. [247] through *in silico* virtual screening approach reported benzamides, a synthetic derivative of flavones, could be promising LasR inhibitors as they exhibit higher binding affinity to the LasR ligand-binding domain compared to the DNA binding domain. Also, the selected compounds conformationally binds to the same amino acid residues of the ligand-binding region similar to the natural ligand, which indicated the competitive nature of the compounds. Mellini et al. [248] attempted a virtual screening on *in silico* FDA-approved drugs library consisting of 1467 compounds through molecular docking and selected five top hit compounds that possessed a stable binding affinity for the QS receptor PqsR as novel antagonists of the *pqs* system which imparts its effect on *P. aeruginosa* PqsR associated expression of virulence factors.

Another report by Shah et al. [249] found a synthetic compound potassium 2-methoxy-4-vinylphenolate to be the most powerful *P. aeruginosa* quorum-sensing inhibitor that targets LasIR/RhlIR circuits and inhibits the formation of biofilm, production of virulence-associated factors like LasA protease, pyocyanin, LasB elastase, and motilities in bacteria. Recently, a study by Nain et al. [250] utilized energy-optimized pharmacophore coupled virtual screening to discover QS inhibitors for LasR of *P. aeruginosa* based on hydrogen bond networking and further exploited the stability of the binding complexes through a dynamics simulation study. Singh and Bhatia [251] performed a study on an FDA-approved clinical drug, Albendazole, by a structure-based molecular docking approach that exhibited putative interactions with LasB and CviR receptor protein of *P. aeruginosa* and *Chromobacterium violaceum*, respectively.

Paczkowski et al. [252] screened a highly diverse library of chemical compounds consisting of 60,000 molecules to identify putative flavone-based QS inhibitors. The structure-activity relationship analysis reported that flavone A ring backbone with two hydroxyl moieties is necessary for LasR/RhlR inhibition and thus stated as flavonoids might function in a non-competitive way to hinder LasR/RhlR DNA-binding by altering the transcriptional regulation of quorum sensing mediated target promoters and thereby leading to a decrease in the production of virulence factors. An *in silico* approach with combined pharmacophore and molecular docking studies was carried out by Xu et al. [253] for screening a library of 167,740 compounds derived from the Specs database for potent QscR agonists and LasR antagonists since QscR is an indirect suppressor and represses the *las* system by binding to the promoter region of LasI or forming an inactive heteromultimer with LasR. Concerning this mechanism, it was suggested that QscR agonists and LasR antagonists can have synergistic effects which led to the identification of four potential compounds as biofilm inhibitors with novel scaffolds.

A report by Jha et al. [254] stated a high throughput computational docking approach to identify an inhibitor for LasR of *P. aeruginosa* and suggested [(4E)-1-hydroxy-3-methylpyridin-4(1H)-ylidene]azinic acid] as the best antagonist of LasR as it satisfied the ADMET profile for drug properties among the top five inhibitors obtained post the screening process. Tan et al. [255] discovered novel QSI candidates using 3040 structures of natural compounds and their derivatives by a structure-based virtual screening (SB-VS) protocol and found five compounds to have the efficiency to block QS-associated expression of genes in *P. aeruginosa* utilizing a live reporter gene assay.

Wei et al. [256] designed a computational network to measure cell–cell interactions directly and dynamics of biofilm at a fundamental level, which revealed the quorum sensing inhibitor (QSI)-based therapies to decrease the spread of QSI resistance through simulations. Sharma et al. [257] reported a web server, dPABBS, which could predict, and design anti-biofilm peptides based on the whole composition of amino acid residues, selected features of residue, and positional preference of amino acid residues to fight *P. aeruginosa* biofilms. Another interesting piece of research by Kim et al. [258] found 6-gingerol from fresh ginger, a pungent oil, antagonizes *P. aeruginosa* QS receptors through hydrogen bonding and hydrophobic interactions with LasR as revealed by molecular docking analysis. Supporting the above information, transcriptomics analysis also proved that 6-gingerol repressed QS-induced genes, particularly virulence factor production.

Using a bio-sensor strain of *P. aeruginosa*, Gopu et al. [259] reported quercetin as a competitive inhibitor of LasR through *in silico* methods, namely molecular docking and simulation studies. A system-level approach was carried out to quantify the capacity of biofilm formation by mutants for identifying the target genes necessary for metabolism in the planktonic state of bacteria. It was seen that the essential gene mutation treatment made *P. aeruginosa* survive by regulating the metabolism of acetate, arginine, and glutamate [260].

Computational methods employ a crucial role in the current drug discovery process starting from designing and maintenance of small molecule libraries, improvisation of pharmacological properties of the lead compounds to the final stage in clinical development. Thus, *in silico* methods could be a productive approach and form alternative tools in all stages of drug development for efficacy, safety, speed with more certainty, and lower cost [261]. On the other side, though several QS inhibitors have been identified so far, much of them do not suit further drug development as lead compounds because of their undesirable pharmacological properties and cytotoxicity [248]. Also, much of the literature on computational approaches have resulted in lead compounds but does not provide further experimental validation of their biological activity [262].

In addition, molecules identified by the virtual screening approach might be false positives resulting from molecule aggregation and could be considered as "promiscuous inhibitors" if they have not been properly investigated by in vitro experimental verifications. For many years, the concept of computational studies has come into play after the synthesis of molecules and a significant illustration of the data. However, now predictions

are made for pharmacological activity without in vitro or in vivo validation. In such a case, it is important to understand predictive models only have a limited prediction domain and they are not very accurate and reliable [263].

8. Conclusions

Members of most *Pseudomonas* species readily form biofilms and remain as a causative organism for biofilm-mediated ailments leading to the development of recurrent infections and chronic infectious diseases. In connection to this, the opportunistic pathogen *P. aeruginosa* in its mucoid state grabs attention in the research area because of its association with biofilm formation. One of the major drawbacks in the treatment of these biofilm-related infections is its wide spectrum resistance to antibiotic treatments that already exist. Hence, in this review, we have summarized the process and mechanisms intricated in biofilm formation by *P. aeruginosa* and the various possibilities so far identified to treat these biofilms effectively. In recent years, several investigations and strategies exploited to study the underlying mechanism of biofilm formation and to control its pathogenesis have been demonstrated. However, significantly more optimal methods utilizing advanced techniques are still needed to identify a more efficient antibiofilm agent to treat *P. aeruginosa*-associated infections, as they are complex and difficult to treat with ease.

Author Contributions: Conceptualization, R.M.; Writing—original draft preparation, A.V., P.V., and M.R.; Writing—review and editing, S.N., S.A. and G.-S.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

QS-Quorum Senisng; EPS-Extracellular Polymeric Substances; eDNA-Extracellular DNA; NIH-National Institute of Health; AI-2-Autoinducer-2; AHL-Acyl Homoseine Lactone; PQS- Pseudomonas Quinolone Signal; IQS-Integrated Quorum Sensing; 3-oxo-C12-HSL-N-(3-oxododecanoyl)-L-homoserine lactone; C4-HSL-N-butanoyl-L-homoserine lactone; WHO-World Health Organization; PLL-Phosphotriesterase-like lactonases; PDMS-Polydimethylsiloxane silicone; CFU-Colony Forming Unit; DspB-Dispersin B; PNAG-Poly-b-1,6-N-acetyl-D-glucosamine; LBD-Ligand Binding Domain; SBVS-Structure Based Virtual Screening; QSI-Quorum Sensing Inhibitor.

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