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## **An Insight Into Beneficial *Pseudomonas* bacteria**

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Additional information is available at the end of the chapter

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### **Abstract**

*Pseudomonas* is a widespread bacterial genus embracing a vast number of species. Various genosystematic methods are used to identify *Pseudomonas* and differentiate these bacteria from species of the same genus and species of other genera. Ability to degrade and produce a whole spectrum of compounds makes these species perspective in industrial applications. It also makes possible to use various media, including wastes, for cultivation of *Pseudomonas*. Pseudomonads may be applied in bioremediation, production of polymers and low-molecular-weight compounds, biocontrol. Recent studies open up new frontiers for further use of *Pseudomonas* in various areas.

**Keywords:** *Pseudomonas* bacteria, physiology, taxonomy, application

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### **1. Introduction**

*Pseudomonas* is one of the most studied species of bacteria. They were first identified at the end of 19th century by Migula as Gram-negative, rod-shaped and polar-flagellated bacteria. Since that time description of genus *Pseudomonas* has widened; development of new methods allowed to study in detail the morphology and physiology of these bacteria. However, the morphological characteristics of *Pseudomonas* are common to many bacterial genera and so are of little value in the positive identification of members of the genus. Advanced nucleic acid-based methods allow to differentiate it from other similar genera and reveal taxonomic relationships among various bacterial species including *Pseudomonas*.

Genus *Pseudomonas* is represented by species that occupy a wide range of niches owing to metabolic and physiological diversity. This diversity allows pseudomonads to adapt to

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challenging environment, resist to adverse conditions caused by abiotic and biotic factors such as high and low temperature, moisture, oxygen and nutrients availability, antibiotics, etc. Elevated resistance provides for ubiquitous distribution of *Pseudomonas* in soil and water, as well as plant growth-promoting rhizobacteria (PGPR), animal and plant pathogens. The bacterium is capable of utilizing a broad spectrum of organic compounds as sources of carbon and energy, hence it is able to colonize habitats where nutrients are limited.

Diversity of *Pseudomonas* determines vast research interest in this genus. Some species like *P. aeruginosa* are opportunistic human pathogens showing enhanced antibiotic resistance, so that studies of pathogenic strains are centered on mechanisms of this antibiotic resistance. Other species are able to degrade a number of compounds that are toxic or recalcitrant to other bacterial species, or produce a wide range of secondary metabolites and biopolymers. It makes these strains perspective for industrial applications.

## 2. Morphology and physiology of *Pseudomonas* bacteria

*Pseudomonas* are Gram-negative, aerobic, motile by one or several polar flagella, non-spore-forming straight or slightly curved rods. In addition to the polar flagella, some species (*P. stutzeri*, *P. mendocina*) have shorter lateral flagella. Solid media favor the formation of lateral flagella which are closely related with swarming of cells on solid surfaces [1]. The number of flagella has taxonomic importance. Most *P. aeruginosa* cells carry only one flagellum, although some cells hold two or three flagella. *P. alcaligenes*, *P. mendocina*, *P. pseudoalcaligenes*, and *P. stutzeri* are also characterized by a single flagellum. The majority of species possess more than one flagella [2].

Some *Pseudomonas* species also form pili (*P. aeruginosa*, *P. alcaligenes*, *P. syringae*). Type IV pili of *P. aeruginosa* similar to pili of other pathogenic bacteria are involved in cell adhesion to epithelial cells [3, 4]. Pili are essential for the normal development of *P. aeruginosa* biofilms, and they also function as receptors for bacteriophage binding [5-7]. The adhesive region is located at the tip of the pilus. Pili of phytopathogenic *P. syringae* serve as a conduit for the long-distance translocation of effector proteins in plant cells [8].

Bacterial cells don't produce prosthecae and aren't surrounded by sheaths, but they can form biofilms that provide attachment of cells to the substrate and increase stability under adverse conditions [9].

Another important *Pseudomonas* feature is production of variety of pigments. Character of pigmentation remains significant factor among the diagnostic traits of *Pseudomonas*. Pigments may be soluble in water and diffusible into the medium or may be associated with the cells. Pseudomonads can produce diffusible pigments that fluoresce in short wavelength (254 nm) ultraviolet light. Some of these pigments, like yellow-green pyoverdine (fluorescein), are siderophores that play an important physiological role in satisfying the iron requirement. The synthesis of pyoverdine is strongly related to iron starvation. It can be demonstrated by cultivating the bacteria in media such as King's medium B. Pyoverdine binds iron (III) ions

very tightly, and that ferripyoverdine complex is actively transported into the bacterial cell [10, 11]. Pyoverdine from *P. aeruginosa* is essential for virulence in animal models [12]. Pyoverdine also can be a tool for identification of *Pseudomonas* because each genomic group is characterized by a specific pyoverdine [13]. Other pigments produced by species of *Pseudomonas* include pyocyanin (*P. aeruginosa*, blue color), pyorubin (*P. aeruginosa*, red color), chlororaphin (*P. chlororaphis*, green color), pyomelanin (*P. aeruginosa*, brown/black color). *P. mendocina* is able to produce carotenoid pigment [14, 15].

*Pseudomonas* are aerobic bacteria, but in some cases they can use nitrate as alternate electron acceptor and carry out denitrification (*P. aeruginosa*, *P. stutzeri*, and some *P. fluorescens* biovars), reducing nitrate to  $N_2O$  or  $N_2$ . Additionally, *P. chloritidismutans* can utilize chlorate ( $ClO_3^-$ ) as an alternative energy-yielding electron acceptor [16].

*Pseudomonas* tends to utilize organic acids in preference to more complex organic compounds. It represses many inducible peripheral catabolic enzymes. Most of *Pseudomonas* species have incomplete glycolytic pathways, lacking 6-phosphofructokinase, therefore sugars and organic acids are dissimilated prevalently via the Entner-Doudoroff pathway. Representatives of the genus can utilize common monosaccharides (glucose, fructose, galactose, l-arabinose), but growth of some species (*P. stutzeri*, *P. mendocina*, *P. syringae*) may be slow. Most hexoses and related compounds are also degraded by the Entner-Doudoroff pathway and various peripheral pathways [2, 14, 17].

Strains of *Pseudomonas* can grow in minimal media with ammonium ions or nitrate as nitrogen source and a single organic compound as the sole carbon and energy source, not requiring organic growth factors. Some species like strains of phytopathogenic *P. syringae* grow very slowly in comparison with strains of the saprophytic species, but their growth can be enhanced by addition of small amounts of complex organic materials (yeast extract, peptones). Significant systematic feature of *Pseudomonas* is inability to accumulate polyhydroxybutyrate, but polyhydroxyalkanoates of monomer lengths higher than  $C_4$  may be accumulated when growing on alkanes or gluconate. Optimal temperature for growth is approximately  $28^\circ C$ , although some species can grow at  $4^\circ C$  or  $41^\circ C$ . Most species can't tolerate acid conditions (pH 4.5 or lower) [14].

Members of the genus *Pseudomonas* are known for their degrading ability on a whole range of substrates, like hydrocarbons, aromatic compounds, and their derivatives. Some of these compounds are natural (toluene, styrene, naphthalene, phenol), other compounds are final products or intermediates from industrial activities (polychlorobiphenyls, dioxins, nitrotoluenes). A considerable number of these compounds is toxic to microorganisms of other groups and to higher organisms. Research revealed 11 central pathways to which many different peripheral pathways converge. Peripheral pathways transform substrates into a few central intermediates (usually dihydroxybenzenes or dihydroxyaromatic acids), which are then ring-cleaved and converted to tricarboxylic acid (TCA) cycle intermediates through the corresponding central pathways. *P. putida* contains 9 out of the 11 identified central pathways, which is in agreement with the wide range of niches that this species can colonize. The ability to degrade aromatic compounds is a strain-specific feature, therefore several pathways that are found in some strains are missing in other strains of the same species [18].

The  $\beta$ -keto adipate pathway is the most widespread *Pseudomonas* pathway of the degradation of aromatic compounds. It includes *ortho*-cleavage protocatechuate (*pca* genes) and catechol (*cat* genes) branches. Both *cat* and *pca* branches are usually present in most organisms, but the *cat* branch is absent in the three available genomes of *P. syringae*. Quinate, *p*-hydroxybenzoate, and phenylpropenoids (*p*-coumarate, caffeate, cinnamate, ferulate, etc.) are degraded via the intermediate protocatechuate. Protocatechuate is cleaved by 3,4-dioxygenase to carboxy-*cis,cis*-muconate that is converted to  $\beta$ -keto adipate enol-lactone by PcaC and PcaD enzymes. The *pca* genes are gathered in a single cluster in *P. fluorescens*, but they are organized in different clusters in other *Pseudomonas* strains [19]. Benzoate, tryptophan, aniline, salicylate, naphthalene, biphenyl, phenol, benzene, toluene, 4-nitrotoluene, and nitrobenzene are degraded via catechol. Benzoate is an intermediate in the catabolism of several aromatic compounds. Its degradation to catechol involves the *benABCD* genes which were identified in the *Pseudomonas* genomes carrying *cat* genes. *Cat* genes encode enzymes involved in catechol *ortho*-cleavage. *CatA*, *catB*, and *catC* encode catechol 1,2-dioxygenase, *cis,cis*-muconate cycloisomerase, and muconolactone isomerase, respectively. The *ben* and *cat* genes are located together in the genomes of *P. fluorescens*, *P. aeruginosa*, *P. stutzeri*, and *P. entomophila*. Reverse situation is observed in most *P. putida* strains.

Genus *Pseudomonas* also displays other metabolic pathways for aromatic compounds: phenylacetyl-CoA (phenylethylamine, phenylethanol, styrene, tropate), homogentisate (phenylalanine, tyrosine), gentisate (salicylate, 3-hydroxybenzoate, *m*-cresol), homoprotocatechuate (4-hydroxyphenylacetate), nicotinate (nicotinic acid), etc [18].

### 3. Taxonomy and identification of *Pseudomonas* bacteria

The genus *Pseudomonas* belongs to phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, family Pseudomonadaceae with type species *P. aeruginosa*. At present the genus includes about 216 species with 18 subspecies and the number of species constantly increases [20].

The identification of *Pseudomonas* is a necessary step preceding further use of these bacteria because of pathogenicity of some strains to plants and animals, including humans, and its wide metabolic diversity. Since the discovery of *Pseudomonas*, a large number of species was assigned to the genus. First classification of *Pseudomonas* species was based on phenotypic characteristics. The most significant work providing phenotypic description of this genus was performed by Stanier et al. Strains of different species were subjected to many phenotypic tests, the most important of which was the nutritional screening [21].

In the 1960s studies on nucleic acid similarity have been started. DNA–DNA hybridization (DDH) has shown high degree of genomic heterogeneity among the species assigned to the genus [14, 22]. DDH is a universal technique that could offer truly genome-wide comparisons between organisms, but it demands large quantities of high-quality DNA (in comparison with PCR-based techniques). It makes DDH time-consuming and labour-intensive [23].

Evidence of the high level of conservatism among ribosomal RNA molecules [24, 25] allowed to divide this genus into five rRNA groups using rRNA–DNA hybridization [26]. Only rRNA group I that included the type species *P. aeruginosa*, all the fluorescent (*P. fluorescens*, *P. putida*, *P. syringae*), and some non-fluorescent species (*P. stutzeri*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. mendocina*) reserved the name *Pseudomonas*. Later the residuary rRNA groups were affiliated to other genera. The species of rRNA group II were transferred to the genera *Burkholderia* and *Ralstonia*, the species of rRNA group III were transferred to the genera *Acidovorax*, *Comamonas*, and *Hydrogenophaga*, the species of rRNA group IV and group V were transferred to the genera *Brevundimonas* and *Stenotrophomonas*, respectively [27–33].

Sequential development of molecular methods has emphasized the role of 16S rRNA in the identification and classification of bacteria, including *Pseudomonas*. Reasons that allow wide use of 16S rRNA for taxonomic studies include: presence in almost all bacteria, often existing as a multigene family, or operons; the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); the 16S rRNA gene (1500 bp) is large enough for informatics purposes [34]. 23S rRNA is excessively conserved and 5S rRNA is too small for research.

As a result of 16S rRNA sequencing by Moore et al., the genus *Pseudomonas* was grouped into 2 distinct intragenetic divisions. These divisions are designated the "*P. aeruginosa* intragenetic cluster" and the "*P. fluorescens* intragenetic cluster" including four (*P. aeruginosa*, *P. resinovorans*, *P. mendocina*, *P. flavescens*) and five (*P. fluorescens*, *P. syringae*, *P. cichorii*, *P. putida*, *P. agarici*) lineages, respectively. Sequence similarities between the species ranged from 93.3% (between *P. cichorii* and *P. cirtonellolis*) to 99.9% (between *P. oloevorans* and *P. pseudoalcaligenes*). It was observed that 148 positions of 16S rRNA were variable among 1492 nucleotide positions, and 65 positions of these nucleotides were located within three hypervariable regions. Approximately 44% of the total gene sequence variability of *Pseudomonas* species occurs in 6% of the 16S rRNA sequence. Regions other than the variable regions are crucial for ribosome functions [35]. In the research of Anzai et al. genus *Pseudomonas* was divided into two clusters using 16S rRNA sequencing. Six groups were defined within the first cluster: *P. syringae*, *P. chlororaphis*, *P. fluorescens*, *P. stutzeri*, *P. aeruginosa*, and *P. putida* groups. *P. agarici* and *P. asplenii* belong to first cluster, but they were not included into any group. The second cluster contained only *P. pertucinogena* group [36].

Although 16S rRNA gene sequencing is useful for classification and identification, it has some resolution problems at the genus and species level. These problematic groups include the family *Enterobacteriaceae* (in particular, *Enterobacter* and *Pantoea*), the *Acinetobacter baumannii*-*A. calcoaceticus* complex, genera *Achromobacter*, *Stenotrophomonas*, *Actinomyces*, and some species such as *Bacillus anthracis*, *Bacillus cereus*, *Bacillus globisporus*, *Bacillus psychrophilus*, *Burkholderia cocovenenans*, *Burkholderia gladioli*, *Burkholderia pseudomallei*, *Burkholderia thailandensis*, *Neisseria cinerea*, *Neisseria meningitidis*, *Pseudomonas fluorescens*, *Pseudomonas jessenii*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus pneumoniae*, etc. Some of these problems are related to bacterial nomenclature and taxonomy; others are related to sequence identity and very high similarity scores. Some species, like *Aeromonas veronii*, could contain up to six copies

of the 16S rRNA gene that differ by up to 1.5% among themselves which might cause identification problems [37].

Some conservative genes such as *gyrB* (DNA gyrase B subunit) and *rpoD* ( $\sigma^{70}$  factor) also can be used for the identification because protein-encoding genes evolve much faster than rRNA genes and provide higher resolution of intrageneric relationships than 16S rRNA sequencing. Using these genes, two major intrageneric clusters were identified. These intrageneric divisions are consistent with the clusters that have been defined using 16S rRNA gene sequence analysis by Moore, but phylogenetic relationships within the clusters differ in comparison with 16S rRNA sequencing. *GyrB* and *rpoD* nucleotide sequences can be also used to design specific PCR primers due to the high evolution rates [38, 39]. *OprI*, *rpoB*, *atpD*, *carA*, *recA*, and *oprF* also can serve as alternative phylogenetic markers when studying *Pseudomonas* taxonomy [40-43].

Another recently introduced method for taxonomic investigations of bacteria is multilocus sequence typing/analysis (MLST/MLSA). MLSA is a molecular typing method that consists of sequencing 400-600 bp long fragments of some housekeeping genes, i.e., genes that are present in most bacteria. MLSA has two important advantages over 16S rRNA sequencing: 1) the higher variability of housekeeping genes as compared to the 16S rRNA sequence and increased length of the total analyzed sequence even allow differentiation of strains; 2) sequencing of some genes reduces the risk that horizontal gene transfer obscures the resulting phylogeny [44]. According to the recent MLSA research (16S rRNA, *gyrB*, *rpoB*, and *rpoD* genes) the genus *Pseudomonas*, as before, is divided into two lineages (*P. aeruginosa* and *P. fluorescens*), which are subdivided into three and nine groups, respectively. The *P. oryzihabitans* group (two species) and the type strains of *P. luteola*, *P. pachastrellae*, and *P. pertucinogena* are the most phylogenetically distant from all other *Pseudomonas* and therefore they aren't included in these lineages [45].

In addition to sequencing of different genes it's possible to use a number of other methods. Restriction fragment length polymorphism (RFLP) is related to the polymorphic nature of the locations of restriction enzyme sites within defined genetic regions. As a result of RFLP, restriction profile is revealed. RFLP procedure is simple in manipulation and it doesn't require sequence information allowing to identify bacteria at species or subspecies level. On the other hand, it's time consuming and requires large amounts of DNA. The method was applied to determine genomovars and biotypes of various *Pseudomonas* species using 16S rRNA or 16S-23S spacer regions [46, 47]. The intergenic 16S-23S internally transcribed spacer (ITS1) regions are less susceptible to selection pressure because of their non-coding function and should have accumulated a higher percentage of mutations than the rRNA genes [46].

It's possible to use polymerase chain reaction-reverse cross-blot hybridization (PCR-RCBH) in detection and identification studies. 16S-23S intergenic spacer region was amplified and used in hybridization assay with specific oligonucleotide probes to fluorescent pseudomonads and certain species of the genus. Positive reactions were observed if studied bacteria at least belonged to genus *Pseudomonas*. It was demonstrated that the identification of pseudomonads by PCR-RCBH is highly specific and less time-consuming than the conventional bacterial culture method [48].

Pulsed-field gel electrophoresis (PFGE) can be used for differentiation and identification of single strains [49, 50]. PFGE is often considered the “gold standard” of molecular typing methods. PFGE has the high discriminatory power, however, this method is time-consuming and labour-intensive, and some point mutations can change banding patterns, resulting in misleading results [51]. Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) is also an effective method for identification of *Pseudomonas* genotypes. ERIC-PCR is quick, easy to perform and cost effective, but it has low reproducibility compared to PFGE [52-54].

As mentioned above, some chemotaxonomic markers like pyoverdines also can be taxonomic tools for the identification of *Pseudomonas*. Strains belonging to a well-defined genomic group produce an identical pyoverdine, and each genomic group is characterized by a specific pyoverdine. The same conclusions are valid for nonfluorescent *Pseudomonas* species and their siderophores. Strains are analyzed by two siderotyping methods: siderophore isoelectrofocusing and siderophore-mediated iron uptake. Correlation between DNA-DNA hybridization and siderotyping data was established. Compared to conventional phenotypic and genomic methods, siderotyping is the fast, accurate, and easy-to-perform technique allowing to identify at the species level. Two siderotyping methods can be improved by mass spectrometric determination of the molecular mass of pyoverdines [13, 55, 56].

Another possible tool for *Pseudomonas* taxonomy is fluorescence spectroscopy. In the study of Tourkya et al. analysis of emission spectra of three intrinsic fluorophores (NADH, tryptophan, and the complex of aromatic amino acids and nucleic acid) allowed to clearly discriminate *Pseudomonas* at genus level from *Burkholderia*, *Xanthomonas* and *Stenotrophomonas*. These results correlate with the classification based on 16S rRNA comparison. Fluorescence spectroscopy also allowed to discriminate *P. lundensis*, *P. taetrolens*, *P. fragi*, *P. chlororaphis*, and *P. stutzeri* species from the others. Clustering of these species is also concordant with data from 16S rRNA gene sequence comparison affiliating the four species to the same *P. chlororaphis* group [57].

There are many methods allowing to identify and classify the *Pseudomonas* genus, but gene sequencing procedures proved the most advanced and sophisticated. Great diversity of genus *Pseudomonas* urges further progress of taxonomic methodology.

#### 4. Criteria for selection of *Pseudomonas* bacteria

As mentioned above, pseudomonads are able to degrade a broad spectrum of compounds. They are also characterized by an enormous biosynthesis capacity resulting in the production of a wide range of secondary metabolites and biopolymers. Ability to degrade and synthesize various substances is a vital technological merit of *Pseudomonas*. It promotes practical interest in various biotechnological processes such as bioremediation, production of polymers, biotransformation, synthesis of low-molecular-weight compounds and recombinant proteins, biocontrol agents [58]. The above-mentioned applications demand formulation of criteria for selection of pseudomonads.

## 5. Safety of *Pseudomonas* bacteria

*Pseudomonas* is known to display a range of pathogenic and toxicological characteristics in regard to humans, animals, and plants. The infections pseudomonads cause to humans are generally opportunistic. Individuals most at risk from *Pseudomonas* infection are the immunocompromised, patients with cystic fibrosis, and patients suffering major trauma or burns. The predominant *Pseudomonas* species isolated from clinical sources are *P. aeruginosa* [59]. *P. aeruginosa* is an opportunistic pathogen that may induce severe infections in humans and other vertebrates. Some *P. aeruginosa* strains, like PA14, also cause disease in a variety of nonvertebrate hosts, including plants, *Caenorhabditis elegans*, and the greater wax moth, *Galleria mellonella* [60]. The other *Pseudomonas* infection cases are rare.

Important feature of *Pseudomonas* is antibiotic resistance. Antibiotic resistance in the bacterial community constantly increases, and more multiple drug resistant strains appear. The best studied organism among pseudomonads is *P. aeruginosa*. The species is known for multiple drug resistance. *P. aeruginosa* has acquired resistance via multiple mechanisms, including production of  $\beta$ -lactamases and carbapenemases, upregulation of multidrug efflux pumps, and cell wall mutations leading to a reduction in porin channels [61].

Antibiotics used to treat *P. aeruginosa* infections have to cross the cell wall to reach their targets. The resistance of *P. aeruginosa* to these antibiotics is connected, first of all, with low permeability of the outer cell membrane and the efficient removal of antibiotics by efflux pumps. The above-mentioned mechanisms are common components of the resistance phenotype for  $\beta$ -lactams, aminoglycosides, and quinolone antibiotics. The agents that break down the outer-membrane permeability barrier (cationic antimicrobial peptides [62] or mutations that create large channels in the outer membrane [63]) make cells more susceptible to antibiotics.

The outer membrane contains proteins (porins) which form water-filled channels for diffusion of hydrophilic molecules. Porins play an important physiological role in the transport of various compounds.  $\beta$ -lactams, aminoglycosides, tetracyclines, and some fluoroquinolones can pass through porin channels [64, 65]. The loss of these porin channels can decrease the susceptibility of *P. aeruginosa* to antibiotics. Approximately 163 known or predicted outer membrane proteins were identified with 64 of these outer membrane proteins grouped into three families of porins [66]. OprF is a major porin of *P. aeruginosa* that forms a majority of small channels and a minority of larger channels [67]. OprF is present in high abundance as a closed conformer, and exists as an open channel only at very low levels. Therefore, it was shown that resistance to  $\beta$ -lactam antibiotics does not seem to involve loss or modification of OprF [68].

Porin OprD takes part in uptake of basic amino acids, small peptides and carbapenems (such as imipenem and meropenem) [69, 70]. Any substitution or deletion within external loop 2 and loop 3 of OprD results in changes of conformation and can cause imipenem resistance. Functional deletion of loop 2 at H729 induced partial resistance to imipenem and meropenem. Imipenem was found to bind to sites in loop 2 to block channel function. Deletion of loops 3 and 4 in OprD also results in failed expression. However, loop 3 is more likely to serve as a

passage channel within OprD for imipenem, but not a direct binding site. Loop 1, loop 5, loop 6, loop 7, and loop 8 are not involved in the passage of imipenem, but either the deletion or amino acid substitutions of loop 5, loop 7, and loop 8 resulted in increased susceptibility to  $\beta$ -lactams, quinolones, chloramphenicol, carbapenems and tetracycline [71-76]. Amino acids including histidine, arginine, and lysine, its analogs, and peptides containing lysine can inhibit the penetration of imipenem in *P. aeruginosa* cells [70]. Culture medium containing basic amino acids significantly increased the minimum inhibitory concentration (MIC) of carbapenems against clinical isolates of *P. aeruginosa* [77].

Polycationic antibiotics, such as polymyxin B and aminoglycosides, and EDTA can pass through outer membrane without porins [78]. They displace divalent cations from lipopolysaccharide (LPS) molecules and destabilize the outer membrane increasing susceptibility to these antibiotics [79, 80]. Overexpression of OprH as a result of mutation or adaptation to low  $Mg^{2+}$  concentrations increases membrane resistance. OprH binds to LPS sites which are occupied by divalent cations and prevents access of polymyxin, gentamicin, and EDTA to these sites [78].

Besides porins, *P. aeruginosa* has numerous and highly efficient efflux mechanisms to resist to antibiotics. Efflux pumps include five superfamilies, based on energy source, the phylogenetic relationship and the substrate specificity. There are five superfamilies: SMR (Small Multidrug Resistance), MET (Multidrug Endosomal Transporter), MAR (Multi Antimicrobial Resistance), RND (Resistance Nodulation Division), and MFS (Major Facilitator Superfamily) [81]. *P. aeruginosa* has efflux systems from all five superfamilies, but the largest number of predicted pumps belongs to the RND family with a total of 12 RND systems including two divalent metal cation transporters [82]. The efflux systems are composed of three protein components: an energy-dependent pump located in the cytoplasmic membrane, an outer membrane porin, and a linker protein which couples the two membrane components together. The 10 RND pumps of *P. aeruginosa* without the metal cation transporters are *MexAB-OprM*, *MexCD-OprJ*, *MexEF-OprN*, *MexXY*, *MexJK*, *MexGHI-OpmD*, *MexVW*, *MexPQ-OpmE*, *MexMN*, and *TriABC*, however, not all of these systems are well studied. These systems provide for intrinsic resistance to a number of antibiotics. Deletion, disruption or overexpression of pumps can make strains more or less sensitive to antibiotics or both effects can be shown (in case of *MexCD-oprJ*) [83].

Additionally, *P. aeruginosa* has a number of  $\beta$ -lactamases that are able to hydrolyze such antibiotics as penicillins, monobactams, cephalosporins, and carbapenems.  $\beta$ -lactamases divide into four classes, each including types that are usually plasmid-mediated or chromosomal [84]. The most common imported  $\beta$ -lactamases of *P. aeruginosa* are penicillinases from the molecular class A serine  $\beta$ -lactamases (PSE, CARB, and TEM families). The most prevalent enzymes of this group belong to the PSE family. Class A extended-spectrum  $\beta$ -lactamases also include enzymes from the TEM, SHV, CTX-M, PER, VEB, GES, and IBC families. Extended-spectrum  $\beta$ -lactamases from the class D, metallo- $\beta$ -lactamases from the class B with four major families (IMP, VIM, SPM, and GIM families), OXA-type enzymes, class A carbapenemases of the KPC family also have been found within *P. aeruginosa*. *P. aeruginosa* has an inducible AmpC cephalosporinase which is similar to AmpC of several members of the *Enterobacteriaceae*. Increasing AmpC production provides for resistance to all  $\beta$ -lactams, except the carbapenems.

However, lack of AmpC increases susceptibility to imipenem and doripenem but not to meropenem. Overproduction of AmpC can occur either by induction of the *ampC* gene or through a process of derepression. Overproduction via induction occurs under the influence of specific  $\beta$ -lactams and  $\beta$ -lactamase inhibitors (cefoxitin, imipenem, and clavulanate), but the process is reversible after removal of the inducing agent. AmpC derepression is related to chromosomal mutations, and therefore concentration of AmpC enzyme remains at an elevated level [83].

Another mechanism of antibiotic resistance is modification of antibiotics such as aminoglycosides. Modifying enzymes phosphorylate (aminoglycoside phosphoryltransferase), acetylate (aminoglycoside acetyltransferase), or adenylate (aminoglycoside nucleotidyltransferase) these antibiotics. Aminoglycoside acetyltransferases (AAC) acetylate compounds such as gentamicin, tobramycin, netilmicin, and amikacin at the 1, 3, 6', and 2' amino groups. Aminoglycoside phosphoryltransferases (APH) inactivate kanamycin, neomycin, and streptomycin by modification of the 3'-OH of these antibiotics. Primary role of some phosphotransferases such as APH(3')-IIb may be participation in metabolism, and resistance to aminoglycosides may be provided fortuitously. Aminoglycoside nucleotidyltransferases (ANT) modify aminoglycosides such as streptomycin and gentamicin. ANT(2'')-I with AAC(6') and AAC(3) are the most common enzymes providing for aminoglycoside resistance in *P. aeruginosa*. Enzymes that modify aminoglycosides can be associated with transposons which additionally carry genes for resistance to other compounds. *aac(3)* and *aac(6')* genes are often associated with transposons or integrons carrying genes for extended-spectrum  $\beta$ -lactamases, metallo- $\beta$ -lactamases or genes encoding other aminoglycoside-modifying enzymes [85].

Antibiotic resistance can be provided by changes in targets. Mutations in genes *gyrA* and *parC* (topoisomerases II and IV, respectively) increase resistance to fluoroquinolones. Mainly changes of *gyrA* especially in the Thr-83 codon provide reduced fluoroquinolone sensitivity in *P. aeruginosa*. Usually mutations in *parC* are found jointly with highly resistant *gyrA* mutants [86-88].

Biofilm-forming ability provides resistance to adverse conditions, like antibiotic tolerance in *P. aeruginosa*. Biofilm bacteria are usually embedded in an extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, and nucleic acid [89-92]. The composition of the matrix depends on the environmental conditions, the age of the biofilm, and the particular *P. aeruginosa* strain forming the biofilm. At least three exopolysaccharides have been shown to be produced by *P. aeruginosa*: alginate, Psl, and Pel. Alginates are linear polyanionic exopolysaccharides composed of uronic acids. These compounds decrease susceptibility of biofilms to antibiotic treatment. The Psl polysaccharide is rich in mannose and galactose and is connected with initial attachment and mature biofilm formation. Pel is a glucose-rich, cellulose-like polymer that plays a role in cell-to-cell interactions [93]. Several mechanisms in biofilm increase resistance to antimicrobial agents. These are binding and sequestration of antimicrobial agents by EPS components, stationary phase or slow growth of cells because of nutrient and oxygen limitation within the depths of a biofilm [94, 95]. Alginate produced by *P. aeruginosa* can retard the diffusion of some antimicrobials (piperacillin, amikacin, gentamicin), whereas others penetrate readily (ciprofloxacin, levofloxacin, sparfloxacin, ofloxacin) [96,

97]. Addition of alginate lyase and DNase increase activity of antibiotics [98]. Biofilms are characterized by the heterogeneity: cells close to the substratum exhibit low metabolic activity and cells on top exhibit high metabolic activity. Antimicrobial agents such as ciprofloxacin, tetracycline, tobramycin, and gentamicin interfere with physiological processes of bacterial cells and specifically kill the metabolically active cells in the top layer of biofilms. Other antimicrobial agents such as colistin, EDTA, and SDS interfere with bacterial membrane structures and kill the cells of the deeper layer [99, 100]. However, a small number of bacteria can survive under simultaneous action of both treatments [99].

Thereby *P. aeruginosa* have many mechanisms allowing to survive negative effects of antibiotics. As a result *Pseudomonas* infections are hard to get rid of.

## 6. Waste as media for growth of *Pseudomonas bacteria*

As mentioned above, *Pseudomonas* can grow in minimal media and can utilize a large variety of organic molecules. It appears attractive to use waste as media for *Pseudomonas* cultivation, biodegradation or production of necessary compounds, hence further experiments were carried out.

Frying oil is produced in large quantities by the food industry and private households. The used cooking oil changes its composition and contains more than 30% of polar compounds depending on the variety of food, the type of frying and the number of cycles used. The utilization of these compounds is a growing problem, arousing expanding interest in the use of waste in microbial transformation [101]. Most of the tested *Pseudomonas* showed satisfactory growth on basal medium with 2% or 4% used olive oil or used sunflower oil. Used olive oil also induced biosurfactant production. Sunflower oil was worse substrate for cell growth and biosurfactant production [102].

Biosurfactants are the surface-active compounds that find use in the cosmetic and food production, healthcare, pulp and paper processing, coal, ceramic, and metal industries. They also may be applied in cleaning of oil-contaminated tankers, oil spill removal, transportation and recovery of crude oil, and bioremediation of contaminated sites. Biosurfactants show advantages over chemical analogs owing to their low toxicity and biodegradable nature. *Pseudomonas* is able to synthesize these compounds from cheap carbon sources such as vegetable oils and wastes from the food industry [58, 103].

*P. aeruginosa* LBI strain was grown on media containing one of residues from soybean, corn, babassu, cottonseed, and palm oil refinery. The soybean soapstock waste was the preferred substrate generating 11.7 g/L of rhamnolipids with the best surface-active properties compared with the products from other oil wastes. Biosurfactant from palm oil waste shows a good emulsification index against kerosene suggesting its potential use for bioremediation [104].

Similar experiments showed that waste motor lubricant oil and peanut oil cake [105], waste frying rice bran oil [106], distillery and whey wastes [107], waste frying coconut oil [108], olive oil mill wastewater [109] and molasses [110] can be used as cheap carbon sources for produc-

tion of biosurfactants by *Pseudomonas*. Additionally, these substrates may help solve waste disposal problem.

Glycerol, cassava wastewater, waste cooking oil and cassava wastewater with waste frying oils were evaluated as alternative low-cost carbon substrates for the production of rhamnolipids and polyhydroxyalkanoates (PHAs) by various *P. aeruginosa* strains. Cassava wastewater with added waste cooking oil provides higher levels of rhamnolipids and PHAs compared with the other carbon substrates [111].

PHAs are composed of medium-chain length (R)-3-hydroxyfatty acids characterized by thermoplastic properties, biodegradability and biocompatibility. They make PHAs suitable for use in the packaging, medicine, pharmacy, agriculture and food industries [58]. Technical oleic acid and waste frying oil were shown to be suitable substrates for PHAs production by *P. aeruginosa* strain NCIB 40045 [112]. Glycerol by-product generated during the production of biodiesel from kitchen chimney dump lard was a better carbon source for PHA synthesis by *P. aeruginosa* JQ866912 as compared with commercial glycerol, sugarcane molasses and glucose. Using this glycerol by-product as a carbon source for PHA production could be both environmentally benign and cost-effective coupling of biodiesel and PHA production [113]. *P. oleovorans* is able to produce PHAs using the residual oil from biotechnological rhamnose production as the sole carbon source. PHAs isolated from *P. oleovorans* are more diverse than PHAs from *Ralstonia eutropha* H16 growing under the same conditions [114]. *P. putida* KT2442 produces PHAs in wastewater from olive oil mills (called alpechín), supplemented with glucose, yeast extract and  $\text{NH}_4\text{Cl}$  [115].

Wastes can be used as media in melanin production. Melanins represent a group of macromolecules, synthesized in living organisms by oxidative polymerization of various phenolic substances in the process of adaption [116]. Melanins act as photoprotectants against UV and visible light, charge transport mediators, free-radical scavengers, antioxidants, metal ion balancers [117]. Melanins find applications in agriculture, medicine, cosmetic and pharmaceutical industries. Some bacteria are able to synthesize these compounds. Marine melanin producer *Pseudomonas sp.* (closely related to *P. guinea*) was incubated in marine broth, vegetable waste from cabbage leftovers supplemented with 1.9 % NaCl to maintain salinity and marine broth - vegetable waste medium blended in 30:70 ratio for melanin production. The sole vegetable waste generated no pigmentation. Marine broth medium demonstrated more melanin production than the marine broth - vegetable waste blended medium ( $5.35 \pm 0.4$  and  $2.79 \pm 0.2$  mg/mL after 72h of incubation, respectively). However, melanin from both sources after purification looked alike in appearance. This study confirms that the pigment can be produced from the cheaper substrates without any functional variation [118].

Another possible waste substrate as fermentation media is animal fleshing, the solid waste produced in large amounts by tanning industry. The studied *P. aeruginosa* strain can digest the media and produce alkaline protease, an industrially important enzyme from waste material. Alkaline proteases have considerable application in leather tanning industry [119]. Strain showed maximum alkaline protease production after 20 hours of incubation at the end of exponential growth phase [120].

*P. aeruginosa* MN7 was found to produce proteases when it was grown in media containing only shrimp waste powder, indicating that it can obtain its carbon, nitrogen, and salts requirements directly from shrimp waste. Protease production increased with increasing concentration of shrimp waste powder and reached a maximum value at 60 g/L [121]. Shrimp shell powder can be used for low-cost production of chitinase and chitosanase showing potential applications in the biocontrol of plant pathogenic fungi and insects. Shrimp shell powder (10 mU/mL) was more suitable as an inducer of chitinase production than squid pen powder (7.2 mU/mL), shrimp and crab shell powder (2.8 mU/mL), katsuobushi from mackerel (<0.1 mU/mL), katsuobushi from bonito (<0.1 mU/mL), and chitin (<0.1 mU/mL) [122].

The potential use of keratinous and chitinous wastes, such as chicken-feathers and shrimp wastes for oil-remediation was shown. Cultures were grown in minimal media with crude oil, or oil supplemented with chicken-feathers or shrimp wastes. The presence of organic wastes, mainly keratinous ones, enhanced the oil-hydrocarbons removal to an extent of 90%. Keratinolytic bacteria were better enzyme producers than the chitinolytic ones, and oil removal in the presence of chicken-feathers was 3.8 times higher than with shrimp wastes, and almost twice, in comparison with oil-only added cultures [123].

Various combinations of agricultural wastes can be tested to promote *P. fluorescence* production. Seven different variants were checked to detect the increased production of *P. fluorescence*. Composition containing rice straw, rice husk, wheat husk, cow dung, coconut water was found to be the optimal substrate for cultivation. The chosen combination also favored a high rate of green pigment production in this medium [124].

Toner waste black powder (TWBP) from copiers and printers is considered to be toxic for environment, and introduction of bacteria can alleviate the problem of TWBP disposal. It was stated that *P. spp.* and *P. aeruginosa* utilize TWBP for growth. TWBP was mixed with soil at different concentrations (2g TWBP + 10g soil, 4g TWBP + 10 g soil, 6g TWBP + 10g soil, 8g TWBP + 10g soil and 10g TWBP + 10g soil) and inoculated in minimal salt medium. Among the various tested TWBP concentrations, 2g TWBP dose provoked significant stimulation of bacterial growth [125].

Tobacco-related processes can release wastes saturated with water-soluble nicotine posing biological and ecological hazard. *P. sp.* ZUTSKD consumed nicotine as sole source of carbon, nitrogen and energy when grown in basic inorganic salt medium. Growth and nicotine degradation were observed at substrate concentrations of 2–5.8 g/L. The strain degraded nicotine completely when the concentration of reducing sugar in TWE (tobacco waste extract) was lower than 8 g/L. Glucose concentration above 10 g/L inhibited nicotine degradation. Yeast extract and phosphate additions improved nicotine degradation in 5% TWE [126].

## 7. Stress resistance of *Pseudomonas* bacteria

*Pseudomonas* species thrive under moist conditions in soil (particularly in association with plants), in sewage sediments and the aquatic environments. Environmental conditions which

will affect their growth include nutrient availability, moisture, temperature, competition, UV irradiation, oxygen availability, salinity and the presence of inhibitory or toxic compounds, but nutritional demands of *Pseudomonas* are modest [59].

There are some ways that allow pseudomonads to resist to adverse conditions. The alternative sigma factors RpoS ( $\sigma^s$ ) and RpoE ( $\sigma^{22}$ ; also referred to as AlgU or AlgT in fluorescent pseudomonads) are involved in bacterial survival under stress conditions. The sigma factor encoded by the *rpoS* gene is known to be important for survival under stressful conditions in several bacterial species. Studies of *rpoS* mutant *P. aeruginosa* PAOI revealed a two- to threefold increase in the rate of kill of stationary-phase cells following exposure to heat, low pH, high osmolarity, hydrogen peroxide and ethanol. However, stationary-phase RpoS-negative cells of *P. aeruginosa* were much more resistant than exponentially growing RpoS-positive cells [127]. RpoS gene also is involved in tolerance to antibiotics in *P. aeruginosa* during the stationary phase and heat stress [128].

The sigma factor AlgU contributes to tolerance towards osmotic, oxidative, and heat stresses in the pathogens *P. aeruginosa* and *P. syringae* [129-133]. AlgU in *P. aeruginosa* also plays part in regulation of biosynthesis of EPS alginate. AlgU is essential for adaptation of plant-associated *P. fluorescens* to osmotic and desiccation stresses [134]. *mucABCD* genes ensure tight control of AlgU activity [135]. The *mucA* gene encoding a transmembrane protein, and *mucB* gene encoding a periplasmic protein are negative regulators of AlgU. Stress conditions destabilize the MucB-MucA-AlgU complex, leading to release of AlgU into the cytosol where AlgU becomes active [136].

Production of some compounds can provide bacterial resistance to adverse conditions. PHA-negative mutants were more sensitive to heat treatment than non-mutated cells. The similar effect was revealed in biofilms of PHA-negative mutants as compared to non-mutated strains [137]. PHA availability enhances the ATP and ppGpp levels, and ppGpp has been shown to induce expression of the *rpoS* gene involved in regulation of stress tolerance [138].

*P. putida* NBAIL-RPF9 can survive under saline shock (1 M NaCl for 1 h) or heat shock (45°C for 20 min). It was identified 13 upregulated proteins and one downregulated protein under heat shock, 6 upregulated proteins under heat tolerance, 11 upregulated proteins under saline shock, and 6 upregulated proteins under saline tolerance. During heat shock, heat stress responsive molecular chaperones and membrane proteins, and during salt stress, proteins upregulated to favor growth and adaptation of the bacterium were revealed. Heat shock chaperones DnaK and DnaJ were expressed under both saline and heat stress. The expression of different classes of proteins under abiotic stress can help this organism to adapt and survive under harsh environmental conditions [139].

Study of *P. aeruginosa* culture exposed to steady-state hyperosmotic stress demonstrated increased gene expression (at least threefold) in cells grown in the presence of 0.3 M NaCl and 0.7 M sucrose. Research revealed that 66 genes changed expression level in response to both stressors [140]. Also 40 of those 66 genes are associated with virulence factor expression, encoding proteins of a type III secretion system (TTSS), the type III cytotoxins ExoT and ExoY, and two ancillary chaperones [141, 142]. It has been shown that *P. aeruginosa* accumulated K<sup>+</sup>,

glutamate, trehalose as cytoplasmic osmoprotectants coupled to major organic osmoprotectant N-acetylglutaminylglutamine amide (NAGGN). Exogenous betaine was found to increase the growth rate and to partially replace NAGGN in osmotically stressed wild-type *P. aeruginosa* cells [143].

Organic solvents are extremely toxic to microbial cells, even at very low concentration. The cell membrane is the primary target for these compounds. Solvents penetrate into and disrupt the lipid bilayer of membrane. Concentration plays a crucial role in determining toxicity of organic solvents. Since Gram-negative bacteria have an additional outer membrane, and Gram-positive bacteria have a single cytoplasmic membrane, it was assumed that Gram-negative bacteria are better equipped to resist to organic solvents. Gram-negative bacteria including some strains of *Pseudomonas* possess various adaptive mechanisms of organic solvent tolerance. There are modifications in cell envelope to increase cell membrane rigidity and decrease permeability, enzymes increasing rate of membrane repair, special solvent-inactivating enzymes, action of efflux pumps, release of membrane vesicles with adhered solvent molecules. These mechanisms help bacteria to overcome the toxic effects of organic solvents [144].

As mentioned above, ability to form biofilm provides resistance to adverse conditions, like antibiotic exposure of *P. aeruginosa*. Biofilm beneficial impact is not limited exclusively to antibiotics. Biofilm cells were found to be more resistant to heavy metals than an equal number of free-floating cells. The degree of increased resistance varied depending on the element. EPS binds heavy metals and retards their diffusion within biofilm, protecting cells from stress [145].

## 8. Application of *Pseudomonas* bacteria

Due to simple requirements of growth conditions and medium composition, capacity to produce and degrade a number of compounds, *Pseudomonas* species are regarded as promising microorganisms in various biotechnological applications. As mentioned above, *Pseudomonas* is able to produce biosurfactants and PHAs characterized by low toxicity and biodegradability for further use in different technological areas. It's possible to apply waste in these processes as low-cost media.

*Pseudomonas* is also an excellent source of various enzymes acting as catalysts in specific biochemical reactions. High efficiency and specificity facilitate introduction of enzymes in diverse industrial processes. Enzymes produced by *Pseudomonas* species can be used in leather processing for dehairing of hides [119, 146], hydrolysis of oils to concentrate the derived fatty acids for medical purpose [147], production of monoacylglycerols and hydrocinnamic esters used in food, pharmaceutical and cosmetic industries [148, 149], manufacturing of detergents [150], production of biodiesel [151], remediation [152], etc.

Another possible application of *Pseudomonas* is bioremediation. *Pseudomonas* is able to remove various toxic pollutants from natural environment. Crude oil is known to alter physical and biochemical characteristics of soil. Petroleum contains numerous components including

alkanes, aromatics, resins and asphaltenes. Action of some *Pseudomonas* cultures was shown to degrade constituents of crude oil, automobile oil effluent, and diesel fuel [153-155]. Moreover, pseudomonads can remove heavy metals released into the environment with industrial and domestic wastewaters. The studies proved that *Pseudomonas* strains are able to dispose of such metals as Cr, Cd, Mn, Fe, Cu, Ni, Pb from wastes [156-158]. Some species possess enormous potential for the detoxification of pollutants containing pesticides and phenols [157].

The textile industry makes extensive use of synthetic chemicals as dyes. A significant proportion of these dyes entering the surrounding media via wastewater is toxic to the environment and humans [159]. Dyes obstruct light penetration and oxygen transfer in water reservoirs. They retain stability and persistence in the environment for a long term [160]. Various physicochemical methods have been used for decolorization of dyes in wastewater, but these methods are distinguished by low efficiency, high cost, limited application scope, and production of recalcitrant wastes [161]. Application of bacteria can solve problems typical to physicochemical methods. It was shown that different *Pseudomonas* species efficiently decolorize and degrade dyes. It's possible to increase decolorization rates by changing cultural conditions. The optimum pH and temperature values for color removal are 7–9 and about 37°C, respectively. Immobilization, anaerobic conditions and addition of some compounds, like yeast extract, promote enhanced decolorization rate. Elevated concentrations of dyes and oxygen decelerate color removal [162-167].

*Pseudomonas* may be used as biocontrol agents that reduce disease severity and promote plant growth. They stimulate growth by several mechanisms. The bacteria can produce some compounds that inhibit spread of plant pathogens. These compounds are siderophores, hydrogen cyanide, pyrrolnitrin, phenazine, 2,4-diacetyl phloroglucinol and lytic enzymes (chitinase,  $\beta$ -1,3-glucanase). The inhibitors can act on pathogens directly like chitinase degrading the fungal cell wall or indirectly like siderophore that binds iron (III) ions in the environment and restrains access of pathogen to these ions. Additionally, *Pseudomonas* provides activation of induced systemic resistance (ISR) or systemic acquired resistance (SAR) in plants. Resistance reveals as oxidative burst which can lead to cell death and prevention of pathogen spreading, changes in cell wall composition, production of phytoalexins and PR proteins [168-173].

## 9. Conclusion

Genus *Pseudomonas* represents a diverse group of bacteria including a large number of species. On the one hand, *Pseudomonas* is characterized by ability to grow in minimal media without growth factors; on the other hand, these bacteria are able to produce and degrade a broad spectrum of compounds. *Pseudomonas* is equipped with several mechanisms allowing to resist and survive under adverse conditions. Currently the genus became the attractive object of intensive research.

*Pseudomonas* can be used in bioremediation allowing to degrade toxic compounds and solve problems concerning utilization of wastes hazardous for environment and humans. It was

shown that 11 central and many different peripheral pathways provide for bacterial degradation of a whole range of compounds. *Pseudomonas* promising application is bioremediation of oil-contaminated environment. Crude oil causes changes of soil valuable properties such as fertility, water-holding and binding capacity, permeability, and bioremediation appears the best way to treat the oil contamination problem.

*Pseudomonas* species are capable of synthesizing both low-molecular-weight compounds (rhamnolipids, enzymes) and polymers (polyhydroxyalkanoates) that are often characterized by better properties than chemical analogs. Their potential usage is manufacturing cosmetics, food, oil refining, leather and paper processing, coal, ceramic, metal industries, agriculture, biodiesel production and medicine. Experiments revealed that agricultural and industrial wastes are suitable substrates for production of biosurfactants, polyhydroxyalkanoates, enzymes, melanin, etc. Application of these substrates will solve problems related to utilization of wastes.

Vast potential of pseudomonads as biocontrol agents was demonstrated. *Pseudomonas* decrease negative influence of plant pathogens by various ways. They can either produce compounds that directly affect pathogens or stimulate development of induced resistance in plants. Summing up, *Pseudomonas* species and their products find applications in various fields primarily because they are capable to utilize a wide range of organic and inorganic compounds.

The recent technological advances in the area of genomics and proteomics are now beginning to lay out important avenue of research focused on the role of *Pseudomonas* bacteria and the molecular mechanisms of their beneficial action.

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