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Pyocyanin as a promising drug in aquaculture

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ABSTRACT

Pseudomonas aeruginosa, the Gram-negative aerobic rod belonging to the family *Psuedomonadaceae* is an aquatic and soil bacterium that can infect a range of organisms, including human. Meanwhile, *Pseudomonas* acts as a potential probiotic for marine finfish and shellfishes and has caused growth inhibition of several pathogens including *Salmonella, Staphylococcus aureus, Vibrio parahaemolyticus, Vibrio harveyi, Vibrio fluvialis, Photobacterium demenselae, Vibrio vulnificus and Aeromonas.* Pyocyanin, a blue redox-active secondary metabolite produced by *Pseudomonas aeruginosa* has been proven to be a safe drug for sustainable aquaculture. It has broad-spectrum activity against different species of bacteria and fungi. Here, we review the applications of pyocyanin, its role in sustainable aquaculture, challenges in Pyocyanin production, the need for synthetic biology production for commercialization, environmental considerations, and potential associated risks.

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1. *Pseudomonas* and pyocyanin

The micro-organisms in artificial as well as natural aquatic environment live as a community that directly or indirectly interact with the eukaryotic occupants, and an improved understanding of the micro-organisms that play diverse roles in the environment and animal health could advance sustainable aquaculture (Murphy et al., 2022). Prebiotics and probiotics in aquaculture promote growth enhancement, immunity-boosting, maintenance of intestinal health, and disease resistance (Arun et al., 2023). *Pseudomonas aeruginosa* is a Gram negative aerobic rod belonging to the family Pseudomonadaceae and is an aquatic- soil bacterium that can infect a range of organisms and an opportunistic human pathogen capable of causing infection in hospitalized patients, immuno-compromised hosts and patients with cystic fibrosis (Driscoll et al., 2007; Lyczak et al., 2000). Though microbes are noted for their pathogenicity, several species of bacteria especially marine bacteria exhibit their exceptional ability to synthesize structurally diverse classes of bioactive secondary metabolites with high biotechnological potential (Andryukov et al., 2019). Many bacterial isolates, which are common members of the non-pathogenic microflora of fish and shellfish culture systems, have been shown to inhibit fish and prawn pathogens *in vitro* (Jayaprakash et al., 2005). Pseudomonads are common inhabitants of the aquatic environment, including shrimp culture ponds and are commonly associated with gills, skin and intestinal tract of live fish (Cahill 1990; Otta et al. 1999). Bathing Atlantic salmon fishes pre-smolts, in a strain of *Pseudomonas fluorescens* reduced subsequent mortality from stress-induced furunculosis (Smith & Devey 1993). *Pseudomonas* spp. acts as a potential probiotic for marine prawns and has caused growth inhibition of several pathogens such as *Salmonella* spp., *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrio fluvialis*, *Photobacterium demenselae*, *Vibrio vulnificus* and *Aeromonas* spp. (Chythanya et al., 2002; Vijayan et al., 2006; Pham et al., 2008). Phenazines are redox-active pigments produced by *P. aeruginosa,* that affect gene expression, metabolic flux, and redox balancing in their producers (Dietrich et al., 2013). The colorful secondary metabolites of *Pseudomonas* sp., produced under conditions of high cell density and nutrient limitation, suggest their importance in the persistence of pseudomonads in the environment (Price-Whelan et al., 2006). Almost all phenazines exhibit broad-spectrum activity against various species of bacteria and fungi (Arunkumar et al. 1997; Kavitha et al. 2005). Phenazines are redox-active compounds and have a role in the generation of reactive oxygen species by *P. aeruginosa* that contributes to the virulence of the organism (Hernandez et al., 2004; Sinha et al., 2015) and have been attributed as signaling molecules in both *P.aeruginosa* PA14 and PAO1(Meirelles and Newman, 2018).

2. Pyocyanin and its Applications

In 1859, Fordos named the blue colored phenazine produced by *P. aeruginosa* as Pyocyanin (‘blue pus’). It was identified as an organic base, blue in alkaline aqueous solutions, but red when acidified. Its chemical reduction to a colorless form and spontaneously reoxidised state in air have been described by Fordos (1860). He established that pyocyanin slowly decomposed to a yellow substance, no longer basic, now known as 1-hydroxyphenazine. Even though the bacterium responsible for pyocyanin production was first isolated by Gessard in 1882 the phenazine nature of pyocyanin could be established by Wrede and Strack in 1924 only. Moreover, they examined its decomposition product, hemipyocyanine, deduced to be 1-hydroxyphenazine. Jensen and Holten (1949) studied the zwitter ion nature of the pigment by way of potentiometric studies and showed that pyocyanin in mixture with its reduced leuco derivative acted as a reversible redox system. At acidic pH, colour changes associated with progressive reduction of pyocyanin are red to yellow to green to colourless, and at alkaline pH the colour change is from blue to colourless (Friedhem, 1931). Cultures of *P. aeruginosa* were observed to reduce

pyocyanin to its colourless form in the absence of air, and depending upon the pH the colour variations account for the shifting play of tints referred to as 'chameleon phenomenon' (Gonçalves and Vasconcelos, 2021).

Pyocyanin is a virulence factor of *P. aeruginosa* and it has its role in the quorum sensing (QS) signaling of the organism (Jayaseelan et al., 2014). Environmental isolates of Pyocyanin producing *P. aeruginosa* have been recognized as putative antagonist against phytopathogenic fungi and bacteria in agriculture and Vibrios in aquaculture (Chythanya et al., 2002; Anjaiah et al., 2003; Bano and Musarrat, 2003; Vijayan et al., 2006). The purified pyocyanin inhibited the growth of *Vibrio* spp. above a concentration of 5 mg/ L (Priyaja et al. 2014). It inhibited the growth and division of a protozoan *Colpidium campylum* as well (Dive, 1973). Pyocyanin exhibits antifungal activity and strong antagonism against *Candida albicans* and *Aspergillus fumigatus* and it was also active against many yeast species pathogenic to humans (Costa and Cusamano, 1975; Kerr et al., 1999). It interferes with the electron transport chain of several fungi and acts as a powerful antifungal agent (Srivastava et al., 2022). Pyocyanin exhibits antibacterial activity against different bacterial species such as *Staphylococci* and *Vibrio* sp. (Arunkumar and Rao, 1997; Anjaiah et al., 2003; Vijayan et al., 2006). Although the steady increase in the antibiotic resistance in almost every pathogen over time is of great concern, not all antibacterial agents exhibit the same rate of resistance development, and new secondary metabolites are continuously required to be added to combat antibiotic-resistant bacterial, fungal and viral diseases (Ramírez-Rendon et al., 2022). The possibility of developing resistance to pyocyanin is very remote as the compound generates reactive oxygen species in the target pathogens leading to their death. Apart from the antimicrobial and antifungal activity of pyocyanin, it may also find application in the preparation of sheets and gown for medical and hospital use and pigment as a textile colourant or dye which can be used to colour cotton, wool and silk fabrics (Srivastava et al., 2022). Pyocyanin is a

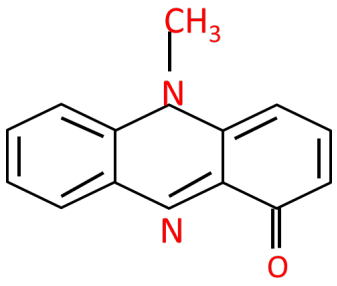

Fig. 1. Structure of pyocyanin

nitric oxide (NO) antagonist in various pharmacological preparations and has various pharmacological effects reported on eukaryotic and prokaryotic cells (Warren et al., 1990; Vukomanovic et al. 1997). An amperometric biosensor system using pyocyanin as a mediator was developed for determination of glucose concentration (Ohfuji et al., 2004). Pyocyanin is used for enhanced power production in a toluene-fed microbial fuel cells that are used to treat xenobiotics-contaminated waste water (Wu et al., 2014). The anti-cancer activity of pyocyanin in HepG2 human hepatoma cells has been studied by Zhao (2014) and the results indicated that pyocyanin accelerated cellular senescence and apoptosis and induced oxidative stress-associated DNA damage in HepG2 cells (Zhao et al., 2014). Evaluation of the physical-chemical and biological properties of pyocyanin unravels the prospects of pyocyanin being applied to different areas of biology, engineering, biotechnology, and medicine (Zhao et al., 2014;Gonçalves and Vasconcelos, 2021).

3. Pyocyanin in Aquaculture

Pyocyanin has been proven as a safe aquaculture drug for the application in RAS-based shrimp culture to control *Vibrio* spp. (Balakrishnan et al., 2022). *P. aeruginosa* isolates from various ecological niches exhibited antagonism to selected pathogenic vibrios in aquaculture (Priyaja, 2012) and pyocyanin has been proven as bacteriostatic at

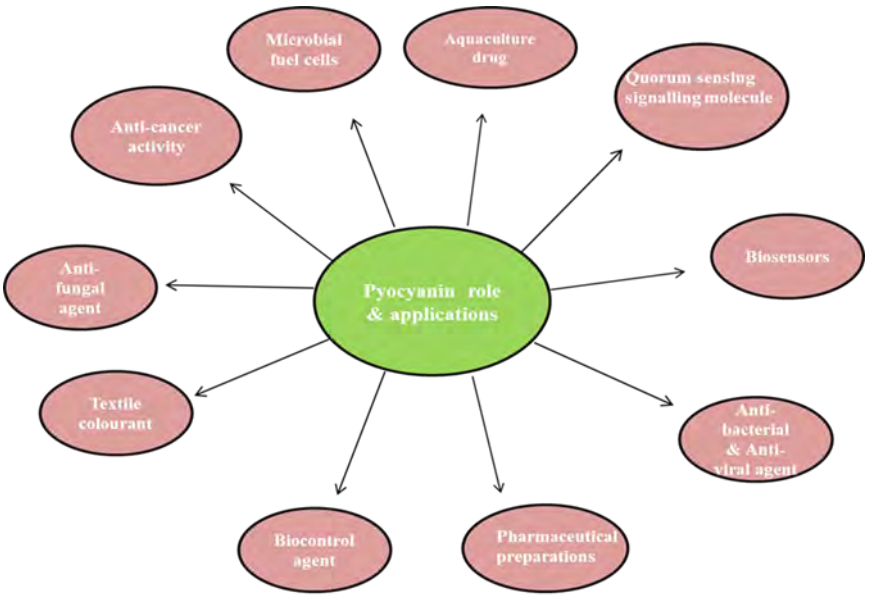

Fig. 2. Applications of Pyocyanin

5 mg L^{-1} and bactericidal at 10 mg L^{-1} and the LC_{50} values of pyocyanin at various life stages of *P. monodon* such as nauplius, zoea, mysis and post-larva were higher than the concentration required to kill pathogenic *V. harveyi.* Meanwhile, pyocyanin did not inhibit nitrifying bacterial consortia used for activating nitrifying bioreactors, at different salinities, in recirculating aquaculture systems suggesting that pyocyanin is a potent as well as safe drug that can be used for sustainable aquaculture (Priyaja et al., 2017). Antagonistic probiotic preparations of *Pseudomonas* MCCB 102 and MCCB 103 could enhance disease resistance and survival of *Penaeus monodon* in larval rearing systems (Pai et al., 2010). Toxicity of the anti-*Vibrio* compound produced by *Pseudomonas* MCCB 102 and MCCB 103 was tested in *Penaeus monodon* haemocyte culture, and the IC_{50} value was found to be 1.4 ± 0.31mg L^{-1} , suggesting its suitability for prophylactic aquaculture applications (Preetha et al., 2009).

4. Genetics of pyocyanin biosynthetic pathway

A detailed study of pyocyanin biosynthetic genes and their corresponding proteins has been carried out by Mavarodi et al. (2001) using expression in recombinant plasmids in a T7 promoter/polymerase expression system and insertional inactivation (Mavrodi et al., 1998a, 2001). *Pseudomonas aeruginosa* contains a complex phenazine biosynthetic pathway consisting of two homologous core loci (phzA1B1C1D1E1F1G1 and phzA2B2C2D2E2F2G2) responsible for the synthesis of phenazine-1-carboxilic acid (PCA), the precursor of pyocyanin (Mavrodi et al., 2001). Mavrodi et al. (2001) reported that these seven genes were sufficient for the synthesis of PCA and two additional genes phzM and phzS encoding unique enzymes were involved in converting PCA to pyocyanin. Knock out mutant analysis on these genes also has proven its contributory effect on pyocyanin production (Chieda et al., 2008; Mavrodi et al., 2001). In *P. aeruginosa*, the operon phzABCDEFG is transcribed as a single mRNA and are localized within a 6.8kb Bg/11-Xbal fragment from the phenazine biosynthesis locus at positions 4,713,795 to 4,720,062 in *P. aeruginosa* PAO1 genome, and a well conserved ribosome binding site preceding each gene (Mavrodi et al., 1998b). phzM gene that spans positions 4,713,098 to 4,712,094 is preceded by a putative ribosome binding site, GAGAGA. PhzS gene, that spans positions 4,720,300 to 4,721,508 located 236 bp downstream of phzG1 of the *P. aeruginosa* PAO1 genome, preceded by a well-conserved ribosome binding site, AAGGAA (Mavrodi et al., 2001). In the seven-gene operon phzE converts chorismate to 2-amino-2-deoxyisochorismic acid (ADIC). Isochorismatase-producing phzD converts ADIC to trans-2, 3-dihydro-3-hydroanthranilic acid (DHHA). The first phenazine nucleus was formed by the condensation of two molecules of trans-2, 3-dihydro-3-hydroanthranilic acid (DHHA) by the products of phzF and phzG genes, and the phzA and phzB stabilize a multienzyme phenazine biosynthetic complex (Mavrodi et al., 1998a). The phzC protein from *Pseudomonas fluorescens* is expressed late in growth and phzC gene could function to divert common carbon metabolites into the shikimate pathway, providing the high levels of chorismic acid needed to support the synthesis of PCA (Mavrodi et al., 1998a). phzM and phzS, which convert PCA to pyocyanin, encode putative phenazine-specific methyltransferase and flavin-containing monooxygenase (Mavrodi et al., 2001). The phzC, phzD and phzE are similar to enzymes of the shikimic acid pathway and along with phzF which play a key role in phenazine synthesis, similar to pyridoxamine-5'-phosphate oxidases, phzG is considered a source of cofactor for the PCA synthesizing enzymes (Mavrodi et al., 1998a). Products of the phzA and phzB genes are highly homologous to each other and may be involved in stabilizing a putative PCA synthesizing multienzyme complex (Mavrodi et al.,1998a). PhzE integrates the nitrogen atoms of phenazines from glutamine (Galbraith et al., 2004; Mentel et al., 2009) .

The phzF exhibits significant structural similarity to the diaminopimelate epimerase (DapF) family of proteins (Parsons et al., 2004). phzM gene encodes a 334-residue protein with a calculated molecular mass of 36.4 kD, and phzS gene encodes a 402-aminoacid protein with a molecular mass of 43.6 kDa and is similar to bacterial monooxygenases (Mavrodi et al., 2001).

5. Biosynthetic pathway of pyocyanin

Pyocyanin biosynthetic pathway involves a cascade of enzymatic reactions that have been well studied. In *P. aeruginosa,* the phenazine biosynthetic pathway branches off from the shikimic acid pathway, which is also the source of metabolites such as aromatic amino acids, siderophores and quinines (Chin-A-Woeng et al., 2001). Usually, the phenazine formation commences after the exponential phase of microbial growth along with associated aromatic amino acid biosynthesis where Shikimic acid acts as a precursor for the simultaneous biosynthesis of phenazines. DAHP (3-deoxy-7phosphoheptulonate) synthase is the first enzyme of the shikimate pathway and catalyses the condensation of phosphoenol pyruvate and erythrose -4-phosphate. The first phenazine structure in the biosynthetic pathway is believed to be phenazine-1, 6-dicarboxilic acid and is formed by the symmetrical condensation of two molecules of chorismic acid (Leisinger & Margraff, 1979). In this step chorismic acid is converted to 2-amino-2-deoxyisochorismic acid

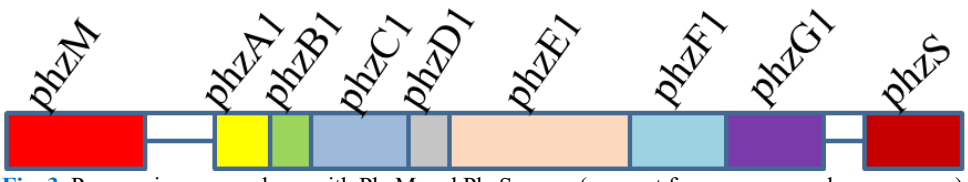

Fig. 3. Pyocyanin operon along with PhzM and PhzS genes (concept from www.pseudomonas.com)

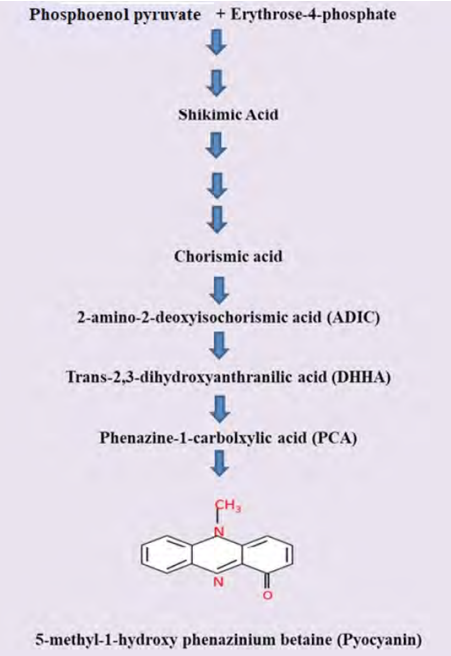

Fig. 4. Biosynthetic pathway of pyocyanin

(ADIC) by aminodeoxyisochorismate (ADIC) synthase and the ADIC formed is converted to trans-2, 3-dihydro-3-hydroanthranilic acid (DHHA). PhzF catalyzes an epimerization and possibly the dimerization reaction that yields the first tricyclic ring structure (Blankenfeldt et al., 2004; Parsons et al., 2004). Pyocyanin is formed from phenazine-1-carboxylic acid by hydroxylative decarboxylation mechanism and the 5-methylphenazine-1-carboxylic acid betaine was shown to be the precursor in this reaction (Parsons et al., 2007).

6. Mode of action of pyocyanin in target cells

Pyocyanin has a unique redox potential, and it accepts a single electron, yielding a relatively stable anion radical that readily undergoes a redox cycle (Hassant and Fridovich, 1980). During respiration, pyocyanin becomes reduced and uni-valently reduces oxygen to the toxic superoxide radical. Hence, the antibiotic action of pyocyanin can be attributed to the production of O^{2-} and H_2O_2 (Hassant and Fridovich, 1980). Hassant and Fridovich (1980) described a mechanism for the toxicity of pyocyanin whereby electron flow from biological pathways is diverted to increase the production of intracellular O_2 reduction products, leading to cell death. Though *P. aeruginosa* is a "strict" aerobe, it is insensitive to pyocyanin and seemingly escapes free-radical injury during the production of or exposure to pyocyanin (Hassett et al., 1991). *P. aeruginosa* is unaffected by the action of intracellular free radicals as there is an increased activity of the enzymes super oxide dismutase and catalase during pyocyanin production (Price-Whelan et al., 2007).

7. Environmental degradation, inactivation, and detoxification of pyocyanin molecule

Environmental degradation of the residual pyocyanin is an important factor as the compound finds application in aquaculture systems. Yang et al. (2007) reported the biodegradation of phenazine-1-carboxylic acid (PCA), the precursor of pyocyanin by the soil organism *Sphingomonas* sp. DP58 which consumes PCA as the sole source of carbon and nitrogen completely degrades it within 40 hours. The presence of phenolic character in the compound favours its degradation by peroxidases, and the oxidation of pyocyanin also leads to its inactivation (Reszka et al., 2004). The study on photosensitized oxidation and inactivation by Reszka (2004) showed that pyocyanin could be partially inactivated through photochemical oxidation and the resulting product (s) is a poorer free radical generator and, therefore, a less efficient stimulant of oxidative processes. Hill and Johnson (1969) reported the microbial transformation of phenazines by *Aspergillus sclerotiorum,* and Chen et al. (2008) conducted a study on intermediates or metabolites produced during PCA biodegradation (Hill and Johnson, 1969; Chen et al., 2008).

8. Challenges in Pyocyanin Production- Methods, needs of synthetic biology

Pyocyanin production is under quorum sensing regulations and the compound is produced only at high cell density and availability of high levels of carbon (Dietrich et al., 2006). Considering the cost of purified pyocyanin and the production cost of the compound, enhancing the production of pyocyanin by its natural host is the need of the hour.

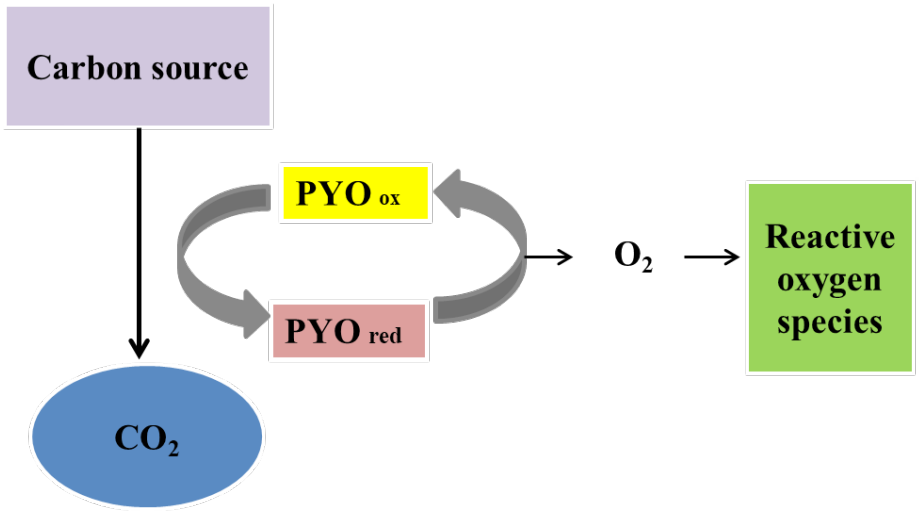

Fig. 5. Mode of action of pyocyanin by the production of ROS

Contemporary approaches employ optimization of culture conditions, regulated batch fermentation systems, medium components, exposure to various chemical and physical factors or usage of genetic engineering methods for strain improvement for enhanced production of pyocyanin (Abdelaziz et al., 2023; Jabłońska et al., 2023). *P. aeruginosa* grown in King's A medium with shaking resulted in increased pyocyanin production (El-Fouly et al., 2015). Optimum yield of pyocyanin was obtained in nutrient broth at 37 ºC and pH 7.2. supplemented with *Illicium verum* extract (Vipin et al., 2017). All these experiments result in microgram scale yield of pyocyanin, which is insufficient for large-scale applications. As mentioned under 'pyocyanin and its applications', pyocyanin is a versatile and multifunctional phenazine, widely used as a bio-control agent. Besides its toxicity in higher concentrations, it has been applied as bio-control agent against many pathogens including *Vibrio* spp. in aquaculture systems. The exact mechanism of pyocyanin production in *Pseudomonas aeruginosa* is well known, but the genetic modification of pyocyanin biosynthetic pathways in *P. aeruginosa* is not much explored to improve the yield of pyocyanin. Synthetic biology tools and techniques (Khalil and Collins, 2010) are relevant in this context. Cell factories are meant for production of native metabolites, bioactive compounds, heterologous expression of biosynthetic pathways, and protein expression (Davy et al., 2017). A detailed study on the possibilities of synthetic biology tools and techniques in the enhanced production of pyocyanin has yet to be carried out.

9. Challenges and Opportunities

Pyocyanin is considered a 'double-edged sword' due to certain negative effects it possesses (Meirelles and Newman, 2018). *P. aeruginosa* is the main cause of nosocomial diseases in immune-compromised hosts and patients with cystic fibrosis and the organism also shows trends of increasing antimicrobial resistance, including carbapenem resistance and multidrug resistance (Driscoll et al., 2007). In eukaryotes, effect of pyocyanin reaches the level of the cell wall or membrane and in the respiratory chain of the mitochondria mitochondria resulting in the release of mitochondrial ROS leading to senescence and apoptosis (Bonifácio et al., 2020; Managò et al., 2015) . Phenazine carboxylic acid, a precursor of Pyocyanin, acts as a signaling molecule that can up-regulate and down regulate several gene expressions in *P. aeruginosa* that affect the survival and virulence of the organism (Du et al., 2015). Pyocyanin plays an important role during biofilm development and also promotes survival within biofilms when cells are oxidant-limited adding to the virulence of the organism (Meirelles and Newman, 2018).Genome level manipulations of *P. aeruginosa* could enhance the biosynthesis of pyocyanin which is a safe aquaculture drug that can be used sustainably. The application of synthetic biology tools would enable the genetic manipulation of the organism. This would shed light on the re-writing of metabolic pathways, the generation of gene constructs, and cell factory development, as part of the economically feasible industrial production of useful compounds.

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