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Computational Analysis of Pyocyanin (*Phz* Gene) From *Pseudomonas*

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Abstract **Original Research Article**

Pyocyanin is a biologically active phenazine produced by *Pseudomonas aeruginosa*. Pyocyanin is a blue redox-active secondary metabolite and a member of the large family of the tricyclic compounds. "Phenazines" Because of its solubility in chloroform it can be easily isolated. Pyocyanin or 1-hydroxy-5-methylphenazine is considered as a resonance hybrid of the mesomeric forms of N-methyl-1-hydroxyphenazine is capable of undergoing a two-electron reduction to a colourless product, leukopyocyanin. It is believed to bestow *P. aeruginosa* with a competitive growth benefit in colonized tissue and is also thought to be a virulence factor in diseases such as cystic fibrosis and AIDS where patients are commonly infected by pathogenic Pseudomonads due to their immunocompromised state. Pyocyanin is also a chemically stimulating compound due to its unusual oxidation-reduction activity. Phenazine-1-carboxylic acid, the predecessor to the bioactive phenazines, is produced from chorismic acid by enzymes determined in a seven-gene cistron in *Pseudomonas aeruginosa*. Phenzine-1-carboxylic acid is whispered to be converted to pyocyanin by the sequential actions of the putative. S-adenosylmethionine dependent *N*-methyltransferase *Phz*M and the putative flavin-dependent hydroxylase *Phz*S. Here we report the Predicted 3D Structure of PHZ S.
Keywords: Pyocyanin, *Pseudomonas aeruginosa,* leukopyocyanin.
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INTRODUCTION

Bacteria of the genus *Pseudomonas* are ubiquitous and are distinguished by their colourful secondary metabolite Phenazine. Phenazine derivatives have been known as redox reagents and the antibiotics which are produced by ample of microorganisms. One of phenazine derivatives, pyocyanin is a blue Phenazine pigment and is the produced by *Pseudomonas aeruginosa*. Pyocyanin (PCN) is synthesized through a series of complex steps mediated by gene products encoded by two phz ABCDEFG operons, and by the *phz*H, *phz*M and *phz*S genes. The genes *phz*M and *phz*S play essential roles in the pyocyanin biosynthesis. Pyocyanin has antimicrobial activity against bacteria, fungi, and protozoa. Due to the biotechnological application of pyocyanin inhibits the growth of crop pathogen and have also been suggested as food colorant pigments.

Phenazine derivatives have been known as the redox reagents and the antibiotics which are produced by several microorganisms. The production and characterization of these compounds were first carried out by Gessard [1]. One of phenazine derivatives, pyocyanin is a blue phenazine pigment and is produced by *P. aeruginosa*. Its chemical structure was elucidated by Wred and Strack [2] and Hillemann [3]. More than 6,000 Phenazine derivatives have been identified and described during the last two centuries. Pyocyanin down regulate the ciliary's beat frequency of respiratory epithelial cells by reducing cAMP and ATP, alter the calcium concentration by inhibition of plasma membrane Ca^{2+} - ATPase, and induce death in human neutrophils. Due to the abundance and biotechnological application of phenazines, pyocyanin have also been suggested as food colorant pigments. Pyocyanin is a nitric oxide (NO) antagonist in various pharmacological preparations, and has various pharmacological effects on eukaryotic and prokaryotic cells. Pyocyanin functions as an electron transfer agent on the membrane of several strains [4].

Biosynthesis of pyocyanin (PCN) and *phz* gene

Pyocyanin (PCN) is synthesized from chorismate through a series of complex steps mediated by gene products encoded by two phzABCDEFG operons, and by the *phz*H, *phz*M and *phz*S genes. In the first step, catalyzed by the SAM-dependent methyltransferase *phz*M, PCA is converted to 5-methylphenazine-1-carboxylic acid betaine. The second step, catalyzed by the NADH (or NADPH)-dependent

flavoprotein monooxygenases. *Phz*S involves hydroxylative decarboxylation of 5-methylphenazine-1-carboxylic acid betaine to pyocyanin. Pyocyanin only when cultures of *E. coli* expressing *phz*S were incubated with filtered extracts from cultures expressing *phz*M. When cultures expressing *phz*M were incubated for 6 h with filtered extracts from cultures expressing *phz*S, only PCA and 1-OHPHZ were detected and it is required for conversion of PCA to pyocyanin and PCN. There are two modifications for the conversion of PCA to pyocyanin: additions of the N-methyl group, converting PCA to 5-methylphenazine-1-carboxylate betaine and hydroxylative decarboxylation of the betaine to form pyocyanin .There are two potential genes in the vicinity of *phzA1B1C1D1E1F1G1* operon that could encode the required PCA- modifying enzymes.

Antibiotic Action of Pyocyanin

Biologically produced pyocyanin was purified by Stephen and his colleague 1981, and the nature of its antibacterial action was determined for several bacteria. The pigment was shown to be bactericidal for all susceptible organisms. Stephen and Hawkey, 2006 indicated that pyocyanin has antibiotic activity against other bacteria and fungi. It is bactericidal for many species including *Escherichia coli*, *Staphylococcus aureus* and *Mycobacterium smegmatis*. Pyocyanin is bactericidal for many species which can exist either in oxidized or reduced form, the latter being an unstable free radical which reacts rapidly with molecular oxygen. This activity may allow *P. aeruginosa* an advantage over competing bacteria occupying the same niche. Furthermore, pyocyanin has a variety of pharmacological effects on eukaryotic and prokaryotic cells [5]. [6, 7] indicated that pyocyanin has antimicrobial activity against bacteria, fungi, and protozoa, but it is little therapeutic value because it is quite toxic to eucaryotic cells, since pyocyanin inhibited mammalian cell respiration disrupt the beating of human cilia, and inhibit both epidermal cell growth and lymphocyte proliferation.

The presence of *phz*M and *phz*S genes is necessary to produce pyocyanin. Regarding the relationship between the production rate of pyocyanin and increased pathogenicity of *P.aeruginosa*, hopefully gathering information about declining factors related to pyocyanin production, disabling this gene or its protein product will assist in curing patients infected with these bacteria. Jamileh *et al.* [8] demonstrated that combination of pyocyanin and colloidal silver nanoparticles demonstrated a strong antibacterial activity against the bacterial strains the increased antimicrobial effects of pyocyanin when mixed with colloidal silver nanoparticles, may enable its use as a hygienic, disinfectant material for hospitals and placemat risk for large numbers of microorganisms. However, an increase in the number of study samples would lead to more accurate results.

Biocontrol

Phenazines (PZs) are well-known pigmented, nitrogen-containing heterocyclic secondary metabolites produced by a variety of bacteria, like *Pseudomonas* [9]. The majority of the work on PZs has focused on the chemical or biological synthesis, redox properties, antitumor, or biological control activities. PZs have been recognized for their antibiotic properties for over 150 years [9]. PZs effectively control a wide range of plant pathogenic fungi and are a well-characterized mechanism of bacterial plant disease control [10-12]. *Pseudomonas* spp. produces three PZ derivatives PCA, 2-OH-PCA and a small amount of 2-OH-PZ [13]. The well-studied opportunistic pathogen of plants and animals, *P. aeruginosa,* produce the methylated PZ derivative pyocyanin (PYO) and a few *P. aeruginosa* strains also produce PCA [14].

Similarly, another well characterized biological control agent, *P. fluorescens* strain 2-79, produces only PCA [13]. The PZs produced by strain 30-84 are responsible for inhibition of *Gaeumannomyces graminis tritici* (Ggt), the take-all fungal pathogen of wheat. PCN was shown to control *Fusarium oxysporum,* the causative agent of tomato foot and root rot. Both PCA and PCN are involved in control of *Pythium myriotylum*, the causative agent of root rot of cocoyam [10]. PYO was shown to inhibit *Septoriatritici* of wheat. More importantly, they compete for colonization sites on the roots of agriculturally important crops, where they thrive as microcolonies (biofilms) and protect the plants from pathogenic fungi. The overall number of organisms competing with *P. fluorescens* for resources does not decline after this strain has colonized the root. This implies that it is not just the antibiotic activity of phenazines that is important for the ability of their producers to compete in the soil [15].

The importance of PZs in various physiological roles is only starting to be realized. An appropriate statement from a review by Laursen and Nielsen [16] states, “little is yet known about the physiological function of phenazines in their natural environment”. Chemical fungicides and pesticides are comprehensively used in current farming practices to protect crops against diseases. However, recently their utilization has been concerned since chemical fungicides and pesticides are highly toxic. They can cause environmental contamination and/or the presence of fungicide/pesticides residues in food products and induce pathogen resistance [17]. Numerous synthetic chemicals may lose their effectiveness as a result of revised safety regulations, concern over no target effects or the development of resistance in pathogen and pest population. Biological control using microorganisms offers an alternative environmentally friendly strategy for controlling agricultural phytophatogens. Chitinases attract the attention as one of the potential candidates for control of phytopathogenic fungi and insect pests. The interest in

chitin degrading enzymes chitinases with biofilm formation and bioactive organism and their application in control of pests and fungal pathogens have advanced significantly, because chitin is a major structural component of exoskeleton of arthropods, insects and fungal cell wall.

However, the production of chitinases enzyme in industries is to be one of the major economic variables, estimated to account for 12 % of the total production cost and is presently uneconomic due to the high prices of the commercially available chitinase [18]. In recent years, there has been a lot of production of chitinase from microorganism. Recombinant DNA technology was much improved for the production of microbial chitinase. But the cost of production is high, so by using the chitin from the fish scale the denaturation of chitin was reduced and the production of chitinase was increased. A more efficient and economically viable process is essential to reduce the cost production of chitinases. The utilization of chitinase in various applications has received attention in biotechnology field. Therefore, investigate the optimization condition on expression of chitinase should be concerned in order to improve its productivity process, maximum its yield and reducing its production cost we used fish scale squander.

Materials and Methods

Extraction of genomic DNA [CTAB/ NaCl solution (10% CTAB in 0.7 M NaCl)]

For extraction of genomic DNA, a standard protocol, CTAB/NaCl method [19] was followed. Extracted genomic DNA was individually stored at 4°C for further studies.

Identification of *P. putida* by 16S rRNA gene

DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of the sequence of all nucleotides. Genotypic identification of strains was done by 16S rRNA sequence analysis. PCR amplification of 16S rRNA gene of *Pseudomonas* sp, was performed to determine the presence of *Pseudomonas* sp. in the template DNA by using the Lane, 1991 and Weisburg *et al.* [20] with universal forward and reverse primers. PCR primers were useful for the amplification of nearly full length small subunit rRNA genes.

Forward primer : 5'-AGAGTTTGATCATGGCTCAG-3',
Reverse primer : 5'-TACGGCTACCTTGTTACGACTT-3'.

Following amplification, all the reaction tubes were stored at 4 °C. A small aliquot of amplified product was run on agarose gel electrophoresis along with the DNA molecular marker 1.5 kbp to analyze the product, according to Sambrook *et al.* [21].

DNA sequencing

The PCR product formed was further purified and sequenced and submitted to NCBI. The DNA sequencing was performed by using forward and reverses primers. A similarity search for the nucleotides sequence of *lap*D gene of the isolate was carried out using BLAST search at NCBI [22]. The 3D structure of the LapD protein was also predicted by using SWISS PRO database.

PCR amplification of *phzS* gene [9]

Amplification of *phz*S gene was done by using the following primers.

Forward primer : 5'-TCTGTCTGTTCCTGGTGGTT-3'
Reverse primer : 5'-CGCAGGTCAATACTGTGAG-3'

A small aliquot of PCR product was run on agarose gel electrophoresis along with the DNA molecular weight marker to analyze the expected amplicon size of 1750 bp units.

DNA sequencing of *phz*S gene

The DNA sequencing was performed by using forward and reverse primers. A similarity search for nucleotides sequence of *phz*S gene of the isolate was done by BLAST search at NCBI [22]. The 3D structure of the *phz*S protein was also predicted by using SWISS PROT database.

PCR amplification of *phz*S gene

In addition to the biofilm formation, many of the species of *Pseudomonas* are capable of producing bio-active compounds. Of various compounds, pyocyanin is an important one, with respect to the PGPR characteristics. Pyocyanin is a fluorescent green colour pigment which is having antimicrobial activity too [13]. Mavrodi *et al.* [9] reported that gene *phz*S for the production of pyocyanin. They also stated that the gene *phz*S is similar to bacterial monooxygenases. Then the 27 isolated *P. putida* is again subjected to the amplification of the pyocyanin gene of our interest. We amplified the gene *phz*S for the presence of an antibiotic compound pyocyanin.

Thomashow *et al.* [13] used *phz*S primers and produced 1215 bp of DNA encoding *phz*S gene from *P. aeruginosa* and crystallized their enzymes responsible for the formation of the gene and relived the importance of the pyocyanin. The genes *phz*M and *phz*S play essential roles in the pyocyanin biosynthetic pathway. Transformation of the PCA-producing strain *P. fluorescens*2-79 with *phz*M and *phz*S triggered the biosynthesis of pyocyanin with large amounts [9].The pathway for biosynthesis of pyocyanin in *E. coli* by mixing induced cultures expressing *phz*Mwith cultures expressing *phzA1B1C1D1E1F1G1* or *phzA2B2C2D2E2FE. coli* expressing *phz*Mand *phz*Sefficiently converted exogenously supplied PCA to pyocyanin. Some of the *P. aeruginosa* strains could not be able to produce pyocyanin, due to lack of *phz* Mand *phz*S genes.

RESULTS AND DISCUSSION

The following results were obtained from the computational analysis of the *phz*S gene.

1 atgagcgaac ccatcgatat cctcatcgcc ggcgccggca tcggcggcct cagttgcgcc
61 ctggccctgc accaggccgg catcggcaag gtcacgctgc tggaaagcag cagcgagata
121 atcgacagcg gcgtcggcat caatatccag ccggcggcgg tcgaggccct catccatcga
181 ctgatgcgcc cggcgctggc ggccaccgcc atccccaccc acgagctgcg ctacatcgac
241 cagagcggcg ccacggtatg gtccgagccg cgcggggtgg aagccggcaa cgcctatccg
301 cagtactcga tccatcgcgg cgaactgcag atgatcctgc tcgccgcggt gcgcgagcgc
361 ctcggccaac aggcggtacg caccggtctc ggcgtggagc gtatcgagga gcgcgacggc
421 cgcgtgctga tcggcgcccg cgacggacac ggcaagcccc aggcgctcgg tgccgatgtg
481 ctggtcggcg ccgacggtat ccattcggcg gtccgcgcgc acctgcatcc cgaccagagg
541 ccgctgtccc acggtgggat caccatgtgg cgcggcgtca ccgagttcga ccgcttcctc
601 gacggcaaga ccatgatcgt cgccaacgac gagcactggt cgcgcctggt cgcctatccg
661 atctcggcgc gtcacgcggc cgaaggcaag tcgctggtga actgggtgtg catggtgccg
721 agcgccgccg tcggccagct cgacaacgag gccgactgga accgcgacgg gcgcctggag
781 gcgatggccg cgttcttcgc cgactgggac ctgggctggt tcgacatccg cgacctgctg
841 acccgcaacc agttgatcct gcagtacccg atggtagacc gcgatccgct gccgcactgg
901 ggccggggac gcatcaccct gctcggcgac gccgcccacc tgatgtatcc gatgggcgcc
961 aacggcgctt cgcaagcaat cctcgacggc atcgagctgg ccgccgcgct ggcgcgcaac
1021 gccgacgtgg ccgcagccct gcgcgaatac gaagaagcgc ggcggccgac cgccaacaag
1081 atcatcctgg ccaaccgaga acgggaaaaa gaggaatggg ccgcggcttc gcgaccgaag
1141 accgagaaga gcgcggcgct ggaagcgatc accggcagct accgcaacca ggtggaacgg
1201 accaccgagg aggtggtcga cctgctcggg cgcggcggct tcgcggtgga gcggatcgtc
1261 cggatcatcg gcgatctgga cgaagccgcc agcctgcggt tgctcggcaa ttgccgcgag
1321 ccacgctag

Fig-1: Nucleotide Sequence of PHZ S gene

>PHZSMNNSNLAAARNLIQVVTGEWKSRCVYVATRLGLADLIESGIDSDETLAAAVGSDAERIHRLMRLLVAFE
IFQGDTRDGYANTPTSHLLRDVEGSFRDMVLFYGEEFHAAWTPACEALLSGTPGFELAFGEDFYSYLKRCPDAG
RRFLLAMKASNLAFHEIPRLLDFRGRSFVDVGGGSGELTKAILQAEPSARGVMLDREGSLGVARDNLSSLLAGE
RVSLVGGDMLQEVPSNGDIYLLSRIIGDLDEAASLRLLGNCREAMAGDGRVVVIERTISASEPSPMSVLWDVHL
FMACAGRHRTTEEVVDLLGRGGFAVERIVDLPMETRMIVAARA

Fig-2: Protein Sequence of PHZ S gene

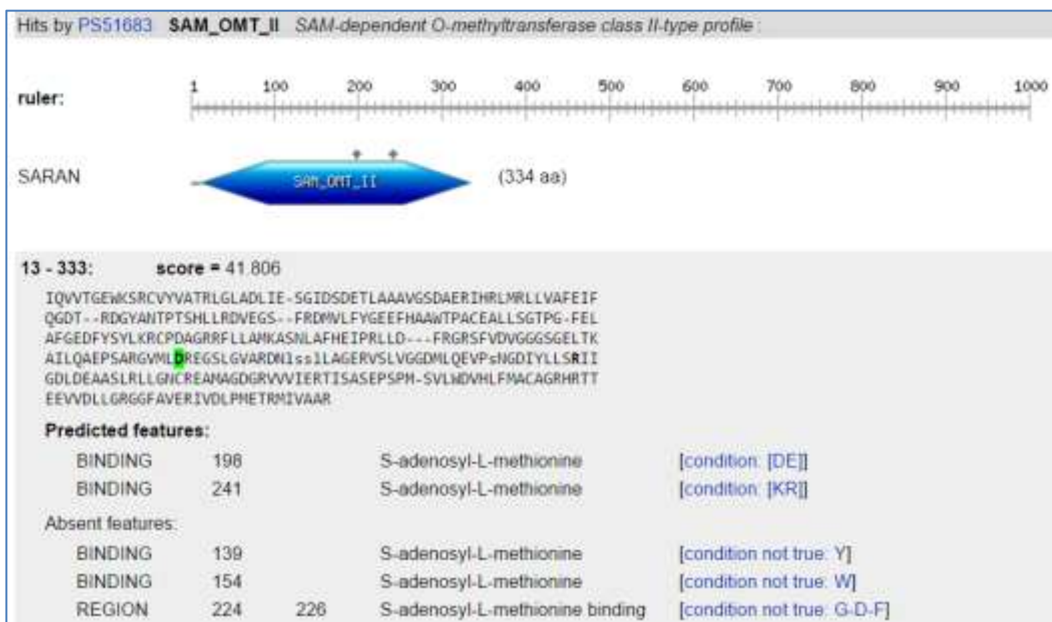

Fig-3: ScanPROSITE prediction of PHZ S

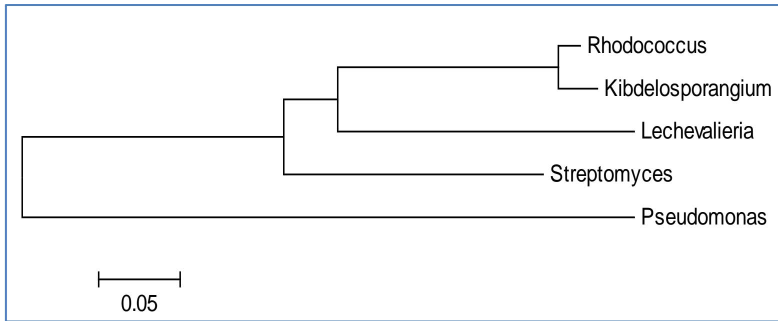

Fig-4: MSA of PHZ S

Fig-5: Phylogenetic tree for PHZ S

Fig-6: Secondary Structure details of PHZ S

Fig-7: Predicted 3D Structure of PHZ S

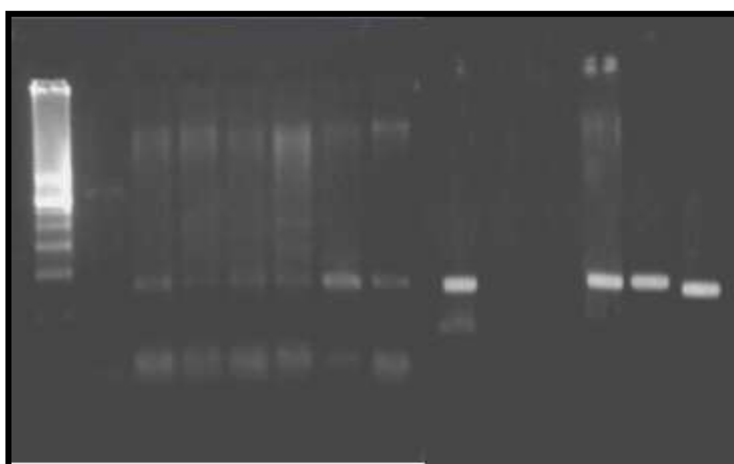

Fig-8: PCR amplification of *phz*S gene

Figure shows the 10 strain gives the positive result of 1215 bp units. Give more explanation on results.

Thomashow *et al*. [13] reported the significant role of *phz*S gene to produce pyocyanin, by comparing with pyocyanin deficient phenotype by inactivating *phz*M gene. In the present study, of 27 isolates tested, 10 of them were positive for pyocyanin. Being an essential bio-component, bacteria with the ability of producing pyocyanin also could be used as biocontrol agent. Pyocyanin has been reported to have bactericidal effect on various plant pathogens, including *Rhizoctoniasolani* [13], *P. chlororaphis* [23]. Pyocyanin producing *P. aeruginosa* environmental isolates have been recognized as putative biological control agents against phytopathogenic fungi and bacteria in agriculture fields [24].

Conclusion

In conclusion, *P. putida* with dual characters, *viz.* biofilm formation and pyocyanin production could be effectively used as plant protection and disease management systems in various agricultural practices. Since all the isolates have been confirmed as non-pathogenic in nature, an effective integrated plant and pest management system could be developed, which is a most-wanted, significant concept required present days. These kinds of formulations are not only supporting the plant growth, but they could biodegrade pesticide residues in soil and nurturing the agricultural soil by providing the needs of soil.

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