An in silico structural, functional and phylogenetic analysis with three dimensional protein modeling of alkaline phosphatase enzyme of *Pseudomonas aeruginosa*

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**Abstract** Phosphorus is a primary macronutrient required for normal plant health, metabolism and survival. It is present in soil in compound insoluble form for which plant cannot uptake it directly from the soil. Some phosphate solubilizing bacteria possess some important enzymes for phosphate solubilization as well as mineralization. Alkaline (or basic) phosphatase (EC 3.1.3.1) is a type of zinc containing dimeric hydrolase enzyme responsible for removing the phosphate groups from various kinds of molecules including nucleotides, proteins, and alkaloids. Unlike acid phosphatases alkaline phosphatases are most effective in an alkaline environment. Alkaline phosphatases (ALPs) are of immense importance in various agricultural industries including dairy industries for testing successful pasteurization process. In this present study, *Pseudomonas aeruginosa* phosphatase was selected for a detailed computational investigation to exploit its physicochemical characteristics, structural properties including 3D model, model quality analysis, phylogenetic assessment and functional analysis using a number of available standard bioinformatics tools. The protein having average molecular weight about 51 kDa, was found thermostable and alkaline in nature belonging to metalloenzyme superfamily. Specifically, the thermostable behavior of the protein is suitable for the dairy industry. Moreover, this theoretical overview will help researchers to get an idea about the predicted protein structure and it may also help to design genetically engineered phosphate solubilizing bacteria by designing specific primers.

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**Abbreviations** ALPs, Alkaline Phosphatases; PSB, Phosphate Solubilizing Bacteria

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1. Introduction

Different bacterial genera are involved in various activities to maintain the soil ecosystem. Phosphorus is a primary plant macronutrient which plays an important role in various plant metabolisms, growth and development. It is not only very important element for functioning as the key enzyme to regulate the different metabolic pathways but also it is the main important structural constituent of nucleic acid. It acts as a main constituent of membrane phospholipid and takes a role in membrane development and its function. It is one of the components of ATP, ADP, and AMP. ATP plays an important role in the cell in all energy requiring process and in metabolic pathways. In addition, phosphorus plays an important role in photosynthesis, respiration, energy storage and transfer, cell division, and cell enlargement. It helps plants to survive winter rigors as well as contributes to disease resistance in few plants [1–3].

A large amount of inorganic phosphate applied to the soil as chemical fertilizer is immobilized rapidly and becomes unavailable to plants [4] as plants can only absorb phosphate in soluble form. The transformation of inorganic phosphate into soluble form is carried out by soil microorganisms. Therefore, phosphate solubilizing bacteria (PSB) have crucial contributions in phosphate cycle, as it helps in both phosphate solubilization and mineralization [5]. One of the phosphate solubilizing enzymes is phosphatase which has been identified in many bacteria such as Pseudomonas, Rhizobium, Azospirillum, Burkholderia, Bacillus, Aspergillus, and Penicillium [1,6]. Phosphatase is an enzyme that removes a phosphate group from its substrate. It is a large group of protein which is present in bacteria, archaea, and eukarya. Mainly two classes of phosphatases are found i.e. acid and alkaline phosphatase. Alkaline phosphatase (Orthophosphoric monoester phosphohydrolase, E.C.3.1.3.1) is one type of hydrolase enzyme. It is a metalloenzyme, the active site of which contains two Zn^{2+} ions and one Mg^{2+} ion per monomer. It plays an important role in phosphate metabolism. The applications of microbial alkaline phosphatase in diverse areas such as immunology, molecular biology dairy technology and diagnostics have been well documented [7–10]. It also plays an essential role in environmental monitoring and it is an important biochemical tool in limnological studies. This enzyme is also very useful for the evaluation of the soil quality and the perturbation occurring in agricultural fields. The demand for enzymes is increasing globally at an Average Annual Growth Rate (AAGR) of 6.3 percent, reaching a value of nearly $7 billion by 2017 [11]. Alkaline phosphatase is having biggest share of $20 of $100 million in the world enzyme market.

Considering the above diverse application of microbial alkaline phosphatase particularly importance in agricultural field the present study is undertaken for its in silico analysis. Pseudomonas aeruginosa is one of the potent phosphate-solubilizing bacteria and used in agricultural field for better uptake of phosphate. It is a gram negative, rod shaped, monoflagellated bacterium used for phosphate solubilization and mineralization in agricultural soil. Complexity of its genome indicates an evolutionary adaptation permitting it to thrive in diverse environments. So, these bacteria have wide application in diversified soil of agricultural field.

In silico analysis of genes and proteins has been receiving greater attention with particular emphasis to find suitable biomarkers for rapid identification of different pathogenic genera [12,13], designing of drugs to combat the pathogenic microbes and superbugs [14,15], diagnosis of infectious diseases [16] and discovery of potent microbial representative useful for several agricultural and animal feed industries [17–19].

In the present study phosphatase enzyme (alkaline phosphatase) and gene sequences of Pseudomonas aeruginosa PAO1 were used for in silico analysis. Attempts were also made to study the physiochemical properties, predict secondary structure, modeling the 3-D protein, evaluate and analyze the alkaline phosphatase of the strain using different standard computational tools to help researchers get more acquainted with the protein structure.

2. Materials and methods

2.1. Retrieval of the sequences

The amino acid sequences of alkaline phosphatase and respective cDNA sequences of those amino acid sequences of 20 different strains of P. aeruginosa were retrieved from NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov) in FASTA format for computational analysis.

2.2. Phylogenetic tree construction

Phylogenetic tree is a branching diagram which helps to understand the evolutionary relationship among the biological species. Here MEGA6 [20–23] software was used to construct the phylogenetic tree. A total of two phylogenetic trees were constructed. One phylogenetic tree of amino acid sequences of alkaline phosphatase of different strains of P. aeruginosa. Another one constituted the cDNA of the protein of different strains of P. aeruginosa [20–23].

2.3. Primary sequence analysis

Any amino acid sequence contains a message which comes from transcription and translation of a gene. In addition, the amino acid sequence bears various important information such as amino acid composition, physiochemical properties such as isoelectric point (pI), molecular weight (Mw), extinction coefficient (EC – quantitative study of protein – protein and protein – ligand interactions), instability index (II – stability of proteins), aliphatic index (AI – relative volume of protein occupied by aliphatic side chains), and Grand Average of Hydropathicities (GRAVY – sum of all hydropathicity values of all amino acids divided by number of residues in a sequence). The physiochemical properties of amino acid sequences of alkaline phosphatase of different strains of P. aeruginosa were analyzed by Expasy protparam tool (http://web.expasy.org/protparam) [24]. Then the amino acid composition of protein of different strains of P. aeruginosa was graphically plotted and analyzed.

2.4. Secondary structure prediction

The secondary structure is related with protein folding. So, the helix, sheet, and turn of amino acid sequences of different
strains of *P. aeruginosa* were predicted by PSI-blast based secondary structure PREDiction (PSIPRED) and Chou and Fasman secondary structure prediction (CFSSP) server (http://cho-fas.sourceforge.net/index.php) [25].

2.5. Protein 3D model prediction

Here the query sequence taken was the amino acid sequence of alkaline phosphatase of *P. aeruginosa* PAO1 (NP_251986.1). So the comparative homology protein model of alkaline phosphatase of *P. aeruginosa* PAO1 was predicted through the SWISS-Model Workspace [26] by picking the most suited template.

2.6. Predicted protein model evaluation and submission

Predicted protein model of alkaline phosphatase of *P. aeruginosa* PAO1 was evaluated and verified from both QMEAN and SAVES server (http://nihserver.mbi.ucla.edu/SAVES), Ramachandran plot, verify 3D [27], and ERRAT [28] were evaluated from SAVES. The model in specified (.pdb) format was submitted to Protein Model Database (PMDB – https://bioinformatics.cineca.it/PMDB/).

2.7. Functional analysis and protein-protein interaction study

To know the interaction of alkaline phosphatase of *P. aeruginosa* with other closely related proteins STRING v10.0 (http://string-db.org/) server [29] was used. Alkaline phosphatase of *P. aeruginosa* PAO1 was selected as query sequence, and functional protein association network was generated. Moreover, the query sequence was also analyzed to determine the family which the protein belongs. For this, motif finder server (http://www.genome.jp/tools/motif/) was used. Active site was predicted from COFACTOR (http://zhanglab.cmb.med.umich.edu/COFACTOR/).

3. Results and discussion

3.1. Sequence retrieval and phylogenetic analysis

A total of 21 amino acid sequences of alkaline phosphatase enzyme of different strains of *P. aeruginosa* were retrieved at first. The amino acid sequences showed the similarity at least 99% and maximum 100% in BLAST. The different strains were *P. aeruginosa* PAO1, *P. aeruginosa* UCBPP-PA14, *P. aeruginosa* VRFP-A05, *P. aeruginosa* PAO579, *P. aeruginosa* HB13, *P. aeruginosa* DHS01, *P. aeruginosa* 2192, *P. aeruginosa* C3719, *P. aeruginosa* VRFP-A06, *P. aeruginosa* PA21_ST175, *P. aeruginosa* MPA01/P2, *P. aeruginosa* CI27, *P. aeruginosa* MPA01/P1, *P. aeruginosa* PADK2_CF510, *P. aeruginosa* CGI1, *P. aeruginosa* E2, *P. aeruginosa* PA45, *P. aeruginosa* VRFP-A02, *P. aeruginosa* VRFP-A03, *P. aeruginosa* VRFP-A07, and *P. aeruginosa* VRFP-A08. In the phylogenetic tree of the amino acid (Fig. 1) sequences of alkaline phosphatase of selected strains of *P. aeruginosa* showed mainly three major groups present. *P. aeruginosa* PAO1, *P. aeruginosa* PAO579, *P. aeruginosa* MPA01/P2, and *P. aeruginosa* MPA01/P1 were very close to each other and they belong to the same group. On the other hand, *P. aeruginosa* HB13 and *P. aeruginosa* PA21_ST175 showed high similarity and therefore remained in the same group. *P. aeruginosa* UCBPP-PA14, *P. aeruginosa* VRFP-A05, *P. aeruginosa* DHS01, *P. aeruginosa* 2192, and *P. aeruginosa* C3719, *P. aeruginosa* VRFP-A06, *P. aeruginosa* VRFP-A07.
Figure 2  Phylogenetic analysis of cDNA of alkaline phosphatase of different strains of *Pseudomonas aeruginosa*.

Table 1  Physicochemical properties of alkaline phosphatase of different strains of *Pseudomonas aeruginosa*.

| Strain                        | Protein accn. No. | Gene accn. No. | aa | PI  | Mw  | II  | AI  | EC  | GRAVY | Half life |
|-------------------------------|------------------|----------------|-----|-----|-----|-----|-----|-----|-------|----------|
| 1. *Pseudomonas aeruginosa PAO1* | NP_251986.1      | 110645304      | 476 | 6.39| 50.41| 33.66| 83.19| 37360| −0.329| 10       |
| 2. *Pseudomonas aeruginosa UCBPP-PA14* | ABJ12547.1      | CP000438.1     | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 3. *Pseudomonas aeruginosa VRFP05* | ESR71710.1       | AXZJ01000068.1 | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 4. *Pseudomonas aeruginosa PAO579* | EJZ73662.1       | ALOF01000013.1 | 476 | 6.39| 50.40| 33.66| 83.19| 37360| −0.329| 10       |
| 5. *Pseudomonas aeruginosa HB13* | ERO87779.1       | AEVV03000001.1 | 476 | 6.39| 50.36| 32.54| 83.40| 37360| −0.322| 10       |
| 6. *Pseudomonas aeruginosa DHS01* | AMA36279.1       | CP013993.1     | 476 | 6.39| 50.39| 32.54| 83.39| 37360| −0.323| 10       |
| 7. *Pseudomonas aeruginosa 2192* | EAZ59425.1       | CH482384.1     | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.326| 10       |
| 8. *Pseudomonas aeruginosa C3719* | EAZ53701.1       | CH482383.1     | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 9. *Pseudomonas aeruginosa VRFP06* | ETD92309.1       | AYSK01000023.1 | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 10. *Pseudomonas aeruginosa PA21_ST175* | EME95396.1       | AOIH01000002.1 | 476 | 6.39| 50.36| 32.54| 83.40| 37360| −0.322| 10       |
| 11. *Pseudomonas aeruginosa MP01/P2* | EHS41813.1       | AKHN01000044.1 | 476 | 6.39| 50.40| 33.66| 83.19| 37360| −0.329| 10       |
| 12. *Pseudomonas aeruginosa CI27* | EKA45482.1       | AKZG01000049.1 | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 13. *Pseudomonas aeruginosa MP01/P1* | EHS38049.1       | AKHM01000014.1 | 476 | 6.39| 50.41| 33.66| 83.19| 37360| −0.329| 10       |
| 14. *Pseudomonas aeruginosa PADK2_CF510* | EIE45322.1       | AJHI01000055.1 | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 15. *Pseudomonas aeruginosa CIG1* | EJY1970.1        | AKBD0100017.1  | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 16. *Pseudomonas aeruginosa E2* | EKA55970.1       | AKZH01000047.1 | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 17. *Pseudomonas aeruginosa PA45* | ENH93030.1       | APMD01000032.1 | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 18. *Pseudomonas aeruginosa VRFP02* | EOQ78370.1       | AOHU01000017.1 | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 19. *Pseudomonas aeruginosa VRFP03* | EQL39513.1      | ATNK01000127.1 | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
| 20. *Pseudomonas aeruginosa VRFP07* | ETD45171.1      | AZBO01000013.1 | 476 | 6.39| 50.42| 32.38| 82.98| 37360| −0.333| 10       |
| 21. *Pseudomonas aeruginosa VRFP08* | ETD43132.1      | AZHU01000142.1 | 476 | 6.39| 50.39| 32.54| 83.19| 37360| −0.328| 10       |
Figure 3  Graphical representation of the difference in composition of amino acids of alkaline phosphatase of different strains of *Pseudomonas aeruginosa*.

Figure 4  Graphical representation of percentage of helixes, sheets and turns of alkaline phosphatase of different strains of *Pseudomonas aeruginosa*. 
Figure 5  Secondary structure analysis of Pseudomonas aeruginosa PAO1.
CI27, *P. aeruginosa* PADK2_CF510, *P. aeruginosa* CIG1, *P. aeruginosa* E2, *P. aeruginosa* PA45, *P. aeruginosa* VRFPA02, *P. aeruginosa* VRFPA03, *P. aeruginosa* VRFPA08 and *P. aeruginosa* VRFPA07 exhibited high similarity index and they belong to the same group. But, *P. aeruginosa* VRFPA07 was mildly different from other strains in this group. In case of phylogenetic tree of cDNA (Fig. 2) all the above mentioned strains of *P. aeruginosa* PAO1 showed 96% similarity with *P. aeruginosa* PAO579. Similarly, phylogenetic analysis of *Bacillus* phytases and *Mesorhizobium* 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase by protein sequences along with their respective cDNA was previously studied by Verma et al.[18] and Pramanik et al. [19] respectively.

### 3.2. Physicochemical characterization

Physicochemical characterization is very important to characterize a specific protein. Table 1 shows the different physicochemical properties of alkaline phosphatase of different strains of *P. aeruginosa*. The total number of amino acid residue of alkaline phosphatase was 476 for all strains. Isoelectric point is the condition of a solution where the amino acid produces the same amount of positive and negative charges and the ultimate charge will be zero. Isoelectric point (pI) of different strains of *P. aeruginosa* for the enzyme was 6.39. So it seemed mildly acidic protein. Almost neutral pH is found to require in *in vivo* condition in contrast to *in vitro* for the optimum activity of the alkaline phosphatase enzyme [30]. The instability indices were between 32.38 and 33.66. As the values of instability index for all strains were lower than 40, it could be a stable protein [18,19]. All strains showed higher aliphatic index which suggested that the protein is thermostable. Here the range of GRAVY laid in between −0.322 and −0.333. Lower range of GRAVY indicated that the better interaction was established in between protein and water [18,19]. The percentage of different amino acids of alkaline phosphatase of different strains presented graphically (Fig. 3). Black circle highlighted portion in Fig. 3 indicated the difference of amount of amino acid components of the protein of selected strains.
Figure 7  Evaluation of protein model form QMEAN and SAVES server.
3.3. Secondary structure prediction

A graphical representation of the percentage of helices, sheets and turns is given in Fig. 4. The secondary structure prediction result shown here indicated that the percentage of alpha helix was much greater than the percentage of other type of conformations of the protein such as sheet and turn. Most of the *P. aeruginosa* strains showed the 63.7% helix. But exceptions were ERF08779.1 and EME95396. *P. aeruginosa* HB13 and *P. aeruginosa* PA21_ST175 showed 64.7% of alpha helix. Percentage of beta sheet was same in all selected strains of *P. aeruginosa* i.e. 54%. The percentage of turn was also almost same in all strains of *P. aeruginosa*. Only ETD45171.1 (*P. aeruginosa* VRFPA07) showed 14.5% turn.
The secondary structure of the amino acid sequence of alkaline phosphatase of \textit{P. aeruginosa \textit{PAO1}} determined elaborately (Fig. 5). No disordered protein binding sites were discovered (Fig. 5). \textit{In silico} characterization including secondary structure prediction of phytase of different \textit{Bacillus} spp. was done by Verma et al. [18], ACC deaminase protein of \textit{Mesorhizobium} by Pramanik et al. [19] and an atypical MAPK phosphatase of \textit{Plasmodium falciparum} was done by Campbell et al. [31]. Roy et al. [32] reported that prediction of secondary structural elements was vital for detection conformational changes within the protein of interest.

3.4. 3D protein modeling, analysis and submission

A previously established well-known template sequence is needed with significant similarity with query sequence to predict the three dimensional structure. Here the selected template sequence was 1ahl.1.A, a crystal structure of a mutant \textit{E. coli} alkaline phosphatase. The sequence identity of the template sequence with query sequence was 73.93%. The resolution of the structure was 2.50 Å. The oligostate of the predicted protein model was homo dimer. The predicted protein model (Fig. 6a–d) of the query sequence was submitted (in .pdb format) in several server to estimate the quality of the model, evaluation and validation. The QMEAN4 (Fig. 7a–h) and QMEAN6 scores are $\leq 1.79$ and $\leq 1.61$ respectively (Fig. 7c, d). The protein model is submitted in SAVES server and the verify 3D score is more than 80%. The verify 3D of the model shows that 95.96% residues have an average 3D-1D score $\geq 0.2$. So, the modeling is acceptable. At least 80% amino acids scored $\geq 0.2$. This protein model showed the ERRAT value 91.533 for both chains (Fig. 7g, h). Analysis of Ramachandran plot revealed that 97.0% residues resided in favored (red) region, while 4.7% residues were in allowed (brown) regions and rest 2.6% residues were present in the generously allowed (yellow) or outlier region. More than 90% residues in favored region are the characteristic of a good quality model [19]. Such type of \textit{in silico} homology modeling was also shown by several workers [17–19,31,33] to predict a variety of 3D protein models of interest. Finally the built model was deposited at PMDB server and the accession number \textit{PM0080564} was obtained.

3.5. Functional analysis

Functional analysis revealed ten potential interacting partners of phoA in the protein interaction network as resolved by STRING analysis (Figs. 8 and 9). The query protein phoA (accn. No.: 110645304), appeared to contain phosphomonoesterase activity. The closest interacting protein having the shortest node was found secA, a preprotein translocase subunit while the distant interacting protein was found to be folB (a dihydroneopterin aldolase) and folE1 (GTP cyclohydrolase), respectively (Figs. 8 and 9). The active site amino acid residues of phoA, as determined using COFACTOR, were found to be Histidine (H), Glutamine (Q), Threonine (T), Alanine (A), Proline (P), Aspartate (D) and Phenylalanine (F). It is also important to note that because of the absence of direct method of detection for detecting the active site amino acids of phoA, the sequence information of the closest homolog 1alkA (PDB ID: 1ALK_A) was used for identifying the active site amino acid followed by sequence alignment analysis between phoA and 1alkA, and then the corresponding active site amino acids for phoA was considered and selected. In addition, two functional motifs were also detected from the functional study in case of phoA which was found to be a member of

![Figure 10](image-url)  
Result of motif finder showing two functional motifs for the alkaline phosphatase protein of \textit{Pseudomonas aeruginosa \textit{PAO1}}.
metalloenzyme superfamily (Fig. 10). In silico functional annotation studies reported previously also considered the functional activities including finding conserved domains, studying protein-protein interactions, and detecting structural motifs [18,19,33].

4. Conclusion

Alkaline phosphatase, one of the key enzymes produced by different microorganisms, plays pivotal role to increase bioavailable phosphorus in soil ecosystem essential for plant growth and development. Thus, understanding the structural properties of this enzyme is important to get further insight into the functional aspect of such important component of phosphate metabolism pathway in microorganisms. Prediction of 3D model of a protein by in silico analysis is a highly challenging aspect to corroborate the data obtained from the NMR or X-ray crystallographic based methods. Therefore, in silico analysis of protein structure is one of the very useful method for studying the structure-function aspects of the protein where the structural data sometimes not readily available because of the non-availability of the crystal structures. In this study, efforts have been made to present an overview of the structure-function aspects of an alkaline phosphatase protein from Pseudomonas aeruginosa in the context of possible functional involvement of this protein in phosphorus metabolism. In this study, a homo-dimeric, thermostable protein, having average molecular weight of 51 kDa was obtained. This protein mainly showed two functional motifs, one of which belongs to alkaline phosphatase superfamily and the other belonging to metalloenzyme superfamily. The thermostable behavior of the protein appears to be important and suitable for dairy industry. This work might be a valuable contribution in the field of Bioinformatics research and may help other researchers to get an idea about the protein structure, its physicochemical properties, structural motifs, and protein-protein interaction essential for agricultural industries, including dairy industry and bioavailability of phosphorus in soil environment. Hence, this work will also help for detection and identification of such type of proteins in vivo or in silico. However, other modeling techniques and in vivo studies would be needed to affirm the claims obtained in the study.

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