

IN SILICO CHARACTERIZATION OF A CUTINASE FROM PSEUDOMONAS FLUORESCENS

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Abstract. *Cutinases (E.C. 3.1.1.74) are hydrolytic enzymes, member of α/β hydrolase superfamily, that degrade insoluble biopolyester cutin, which is the structural component of plant cuticles. Because of the multifunctionality of cutinase enzymes, they are used in several fields such as in food, agriculture, detoxification, dairy product, textile, laundry, chemicals, and pharmaceutical industry. Cutinases are produced by phytopathogenic fungi and bacteria. In contrast to fungal cutinases, relatively little is known about these enzymes from bacterial sources. The role of cutinase has been shown to facilitate the mode of entry of PGPR (Plant Growth-Promoting Rhizobacteria) endophytes into the newly emerged lateral roots or utilized root hairs. PGPR have emerged during the last decades as a potent alternative to chemical fertilizers, pesticides, and other supplements in an eco-friendly agriculture. Among these bacteria that have an agricultural interest, *Pseudomonas fluorescens* is the subject of particular attention. The aim of the study was to better understand and identify the three-dimensional (3-D) structure and properties of cutinase protein from *P. fluorescens* by using bioinformatics tools. Basic physicochemical characteristics were analyzed using Expasy's ProtParam and ProtScale tools. Several physicochemical characteristics of the cutinase enzyme were demonstrated. The instability index value indicated that the protein was highly stable. Furthermore, no transmembrane domain and no peptide signal were found in the protein, and the enzyme has a cytoplasmic localization. Secondary structure analysis was carried out by both SOPMA and PSIPRED programs that revealed that random coil dominated among secondary structure elements followed by α -helix, extended strand, and β -turn. Moreover, tertiary structure prediction was analyzed by Phyre² server based on homology modeling and the predicted model was refined by ModRefiner and validated using PROCHECK'S Ramachandran plot. Ramachandran plot analysis showed that 89.2% of amino acid residues are within the most favored regions. Prediction of enzyme binding site by COFACTOR server was confident with a BS-score > 0.5. Protein-protein interaction networks demonstrated that cutinase of interacted with seven other proteins in a high confidence score. Overall, this in silico analysis study provides insights about physicochemical properties, structure, and function of cutinase from *P. fluorescens*, which would help to produce the enzyme on a large scale allowing its wide exploiting in various industrial and agricultural products and processes.*

Keywords : *Cutinase, Pseudomonas fluorescens, in silico analysis, homology modeling.*

INTRODUCTION

The cutin is an insoluble lipid-polyester composed of hydroxy and hydroxyepoxy fatty acids that constitutes a major constituent of the cuticle (DUTTA ET AL., 2009). Cuticle protects most

of aerial plant organs, such as flowers, leaves, fruits, and stems, from dehydration, and is a barrier to infection by pathogens (CHEN ET AL., 2013). Cutinases (E.C. 3.1.1.74) are inducible extracellular enzymes secreted by fungi, oomycetes, and bacteria that are capable of degrading plant cell walls. They catalyze the degradation of those polyesters, resulting in the release of cutin monomers (CHEN ET AL., 2008). These enzymes are serine esterases that belong to the α/β hydrolase superfamily and present in the Ser-His-Asp catalytic triad (MARTÍNEZ AND MAICAS, 2021). Due to the multifunctionality of the cutinase enzymes (hydrolyzing activity of both soluble esters and lipids), cutinases have been suggested for a broad range of applications, i.e., in food, agriculture, detoxification, dairy product, textile, laundry, chemicals, and pharmaceutical industry (QAMAR AND ALI, 2021). Indeed, cutin-containing agricultural waste products such as apple pomace and tomato peel have been shown to represent low cost inducers for the commercial production of bacterial cutinase via fermentation (FETT ET AL., 2000).

Several bacterial cutinases have been expressed and characterized from the phyllospheric fluorescent *Pseudomonas putida*, the strain of plant epiphyte, *Pseudomonas mendocina*, and the nitrogen-fixing bacterium, *Corynebacterium* sp. (RHEE ET AL., 2006). Moreover, cutinase facilitates the mode of entry of PGPR (plant growth-promoting rhizobacteria) endophytes into the newly emerged lateral roots or utilized root hairs (OLWE ET AL., 2020). During the last decades, PGPR have emerged as a potent alternative to chemical fertilizers, pesticides and other supplements in an eco-friendly agriculture (KUMARI ET AL., 2019). Multiple *Pseudomonas* species are recognized for their beneficial plant role, known as PGPR, and are mainly found within isolates and species from the *Pseudomonas fluorescens* complex of species (GARRIDO-SANZ ET AL., 2021). In addition, members of the *P. fluorescens* complex have been isolated from diverse habitats, including rhizosphere soil (BAKLI AND ZENASNI, 2019), water (WONG ET AL., 2011), plant tissues (RAMAMOORTHY ET AL., 2002), animals (VELA ET AL., 2006), and humans (SCALES ET AL., 2015).

Cutinases are present in both fungi and bacteria; however, previous studies have focused more on fungal cutinases than in bacteria. Although, cutinases are found in Carbohydrate Esterase family 5 (CE5) which today contains over 3000 entries within the Carbohydrate-Active Enzymes database (CAZy, www.cazy.org) (NOVY ET AL., 2021). Only about 106 three-dimensional cutinase protein structures are available in the Protein Data Bank (PDB, www.rcsb.org) including 32 from bacteria. In this context, experimental methods of 3D protein structure determination are of tedious, time-consuming and expensive (BAKLI ET AL., 2020). Therefore, bioinformatics tools are of great interest and are widely applied for the prediction of 3D protein structure during the last decades in several cases (DAHLSTRÖM, 2015; HODA ET AL., 2021).

The present study was aimed to utilize bioinformatics tools for the characterization of cutinase enzyme from *Pseudomonas fluorescens* for its physicochemical characteristics; primary, secondary, and tertiary structure of protein; and functional analysis including protein–protein interaction.

MATERIALS AND METHODS

1. Sequence retrieval

Primary protein sequence of the cutinase from *Pseudomonas fluorescens* S613 was retrieved in FASTA format (accession no. A0A0P8X0D6) from UniProt (Universal Protein Resource (<https://www.uniprot.org>)) and served as query sequence for all analyses.

2. Analysis of physicochemical properties

The determination of different physicochemical properties of cutinase protein sequence including, molecular weight, theoretical isoelectric point (pI), aliphatic index (AI), instability

index (II), number of positive and negative charged residues (R+/-), extinction coefficient (EC), estimated half-life, and grand average of hydropathicity (GRAVY), was computed using ExPasy ProtParam tool analysis (<http://expasy.org/tools/protparam.html>) (Gasteiger et al., 2005). The hydropathy of amino acid sequence of cutinase was predicted utilizing the program of based on Kyte and Doolittle scale (<https://web.expasy.org/protscale/>) (Kyte and Doolittle, 1982).

3. Subcellular localization, solubility prediction, and peptide signal prediction

The subcellular location of the cutinase protein was predicted by CELLO v.2.5 (<http://cello.life.nctu.edu.tw/>) (YU ET AL., 2006). In addition, the SOSUI server (<http://harrier.nagahama-i-bio.ac.jp/sosui/>) (HIROKAWA ET AL., 1998) was run to identify the transmembrane helices in the cutinase protein sequence. On the other hand, SignalP-5.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) was used to search the presence of signal peptide and to determine the protein localization (ARMENTEROS ET AL., 2019).

4. Secondary structure analysis

Secondary structure analysis including number of α -helices, β -turn, extended strand, β -sheet, and coils of retrieved amino acid sequence of cutinase from *P. fluorescens* was carried out using the Self-Optimized Prediction Method with Alignment (SOPMA) from the Network Protein Sequence Analysis (NPS@) server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) (GEOURJON AND DELEAGE, 1995) and PSI-blast-based secondary structure PREDiction, PSIPRED 4.0 (<http://bioinf.cs.ucl.ac.uk/psipred/>) (MCGUFFIN ET AL., 2000).

5. Tertiary structure analysis, refinement, and validation

The cutinase protein sequence from *P. fluorescens* in FASTA format was submitted to web server based Protein Homology/analogy Recognition Engine V2.0 (Phyre²) (<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>) for protein modeling and prediction of the three-dimensional structure (KELLEY ET AL., 2015). The ModRefiner (<http://zhanglab.ccmb.med.umich.edu/ModRefiner/>), which is a high-resolution protein structure refiner, was used to improve the physical quality of the predicted structure (XU AND ZHANG, 2011). Model quality of predicted and refined 3D structure was evaluated by a Ramachandran plot using PROCHECK analysis tools (<https://servicesn.mbi.ucla.edu/PROCHECK/>) (LASKOWSKI ET AL., 1993). Visualization and optimization of graphics of 3D structure was executed by the PyMOL Molecular Graphics System, Version 2.3 (DELANO, 2019).

6. Functional analysis

The interacting proteins involved in cutinase enzyme from *P. fluorescens* were displayed by STRING (search tool for the retrieval of interacting genes/proteins) database version 11.5 (<https://string-db.org>). Moreover, the query sequence of cutinase was also analyzed to determine the family which the protein belongs. For this, MOTIF finder search tool (<http://www.genome.jp/tools/motif/>) was used. COFACTOR server (ROY ET AL., 2012) (<http://zhanglab.ccmb.med.umich.edu/COFACTOR/>) was employed for the prediction of the binding site (active site) in the generated model of cutinase based on its structure, sequence, and protein-protein interaction.

RESULTS AND DISCUSSIONS

1. Sequence retrieval

The amino acid sequence of cutinase enzyme from *Pseudomonas fluorescens* was retrieved from NCBI database in FASTA format (Accession no. GI, 939142558) and was then

characterized using UniProt tool (Accession no. A0A0P8X0D6) (**Table 1**). The protein was predicted to contain 332 amino acids, possess a molecular weight of 36.81 kDa, and has a AB hydrolase-1 proteic domain from the position 61 to 309 with hydrolase activity.

Table 1

Characterization of retrieved sequence of the cutinase protein from *P. fluorescens*

Accession number (GI)	Number of aa	Molecular weight (kDa)	Domain
939142558	332	36.81	AB hydrolase-1

2. Analysis of physicochemical properties

Several physicochemical properties of the cutinase protein were estimated by ExPASy ProtParam tool, like isoelectric point (pI), number of positive and negative amino acids (R+/-), extinction coefficient (EC), instability index (II), aliphatic index (AI), estimated half-life, and Grand Average of Hydropathicity (GRAVY), and are given in **Table 2**.

Table 2

Physicochemical properties of the cutinase protein from *P. fluorescens*

Accession number (GI)	pI	- R	+ R	EC	II	Half-life	AI	GRAVY
939142558	6.43	37	33	60515	36.32 (Stable)	30 hr	78.83	-0.338

pI: Theoretical Isoelectric point, +R: number of positive charged residues (Arg + Lys), -R: number of negative charged residues (Asp + Glu), EC: Extinction coefficient at 280 nm, II: Instability index, AI: Aliphatic index, GRAVY: Grand average of hydropathicity.

The isoelectric point (pI) of a protein indicates the net charge of a given protein (positive or negative) under physiological conditions, which in turn acts as a good indicator for the solubility of the protein at a given pH (MOHAMAD SOBRI ET AL., 2020). On the basis of pI value, the protein was found to have moderate acidic nature. The value of instability index (II) for the protein sequence was less than 40, indicating that the protein is stable.

The aliphatic index (AI) describes the relative volume of a protein occupied by its aliphatic side chains in which the higher the values, the most thermally stable the protein is predicted to be (Mohamad Sobri et al., 2020). In this case, AI value was found to be 78.83, which means that the protein is thermostable. Moreover, the Grand average of hydropathicity (GRAVY) value represents the protein-water interactions. The GRAVY value was found to be negative, indicating the hydrophilic nature of the enzyme. ProtScale analysis tool in turn led to prediction of minimum and maximum hydrophobic position and score for cutinase sequence predicted (**Table 3**).

Table 3

Hydrophobic score and position of the cutinase protein from *P. fluorescens* using ProtScale tool

Accession number (GI)	Position		Score	
	Min	Max	Min	Max
1220446069	204	66	-2.967	2.144

3. Subcellular localization, solubility prediction, and peptide signal prediction

The subcellular position of cutinase from *P. fluorescens* was predicted by different tools. CELLO program predicted that the enzyme was cytoplasmic with the highest reliability of 0.901. Furthermore, SOSUI is a functional analysis tool which distinguishes between membrane and soluble proteins from amino acid sequences and predicts the transmembrane helices. Any hydrophobic portion of the protein is labeled as transmembrane region. Thus, SOSUI predicted the cutinase sequence as non transmembrane soluble protein. SignalP suggested no signal peptide.

4. Secondary structure prediction

The secondary structure of the protein was predicted by both PSIPRED and SOPMA programs. According to SOPMA estimation, the random coil was found to be the most predominant (40.96%) one followed by α -helix (40.66%), extended strand (14.16%), and β -turn (4.22%). The secondary structure using SOPMA was predicted by using default parameters (window width, 17; similarity threshold, 8; and number of states, 4) (Table 4).

Table 4

Prediction of secondary structure of the cutinase from *P. fluorescens* by SOPMA tool

Accession number (GI)	α -helix	Extended strand	β -turn	Random coils
939142558	40.66%	14.16%	4.22%	40.96%

Secondary structure of the cutinase protein was also predicted by PSIPRED 4.0 as shown in Figure 1. This PSIPRED secondary structure prediction result confirmed that of SOPMA and had a high confidence of prediction.

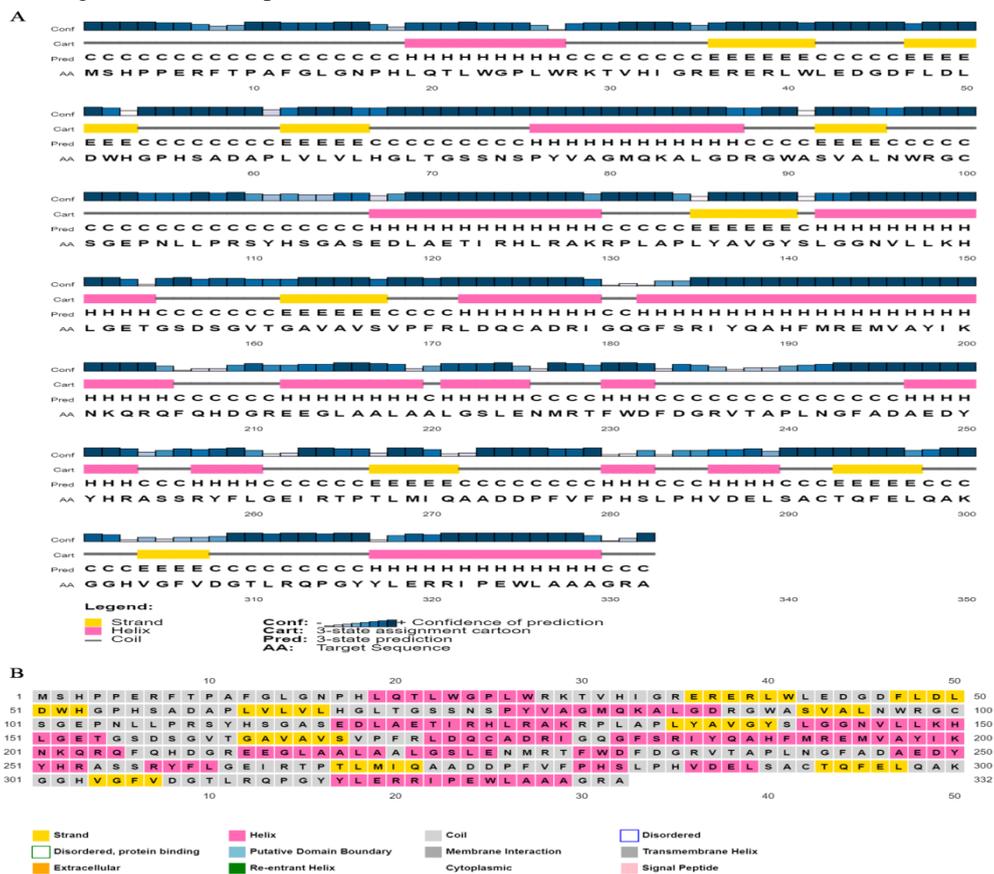


Fig. 1. Secondary structure prediction of *P. fluorescens* cutinase by PSIPRED 4.0 server.

(A) Graphical representation. (B) Sequence annotation plot. The colors represent protein secondary structure elements (yellow for β -strands, pink for α -helix and grey for coil structures). The confidence of prediction observed throughout the predicted secondary structure was high, indicating high reliability of this prediction.

5. Tertiary structure analysis, refinement, and validation

The analytic results of Phyre² software indicated that the secondary structure of cutinase showed identity 14%, 296 residues (89% of the cutinase protein sequence coverage) have been modeled with 100.0% confidence by the single highest scoring template, the crystal structure of gastric lipase (pdb, d1k8qa). This template belongs to α/β hydrolase superfamily. The 3-D structure of the cutinase was successfully analyzed by Phyre² tool and visualized by PyMOL (Figure 2).

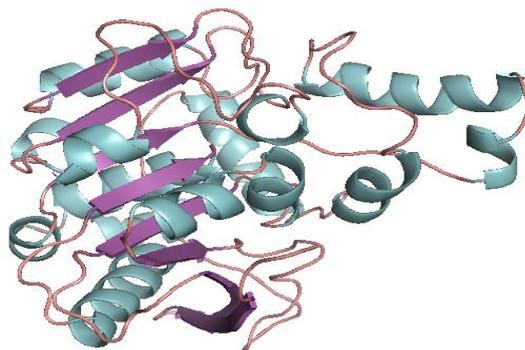


Fig. 2. Predicted 3D structure of cutinase from *P. fluorescens* produced by Phyre², refined by ModRefiner servers and visualized by PyMOL 2.3 molecular graphics software. Secondary structure prediction of Gtf3 protein showing α -helix (green), β -sheets (pink), and loops (orange).

Homology modeling was the most accurate computational method to generate reliable structural models. It was used in many biological applications. Model quality assessment tools were used to estimate the reliability of the models. The stereochemical quality of the predicted model and accuracy of the protein model was evaluated after the refinement process using Ramachandran map calculations computed with the PROCHECK program. The assessment of the predicted model generated by Phyre² was shown in Figure 3.

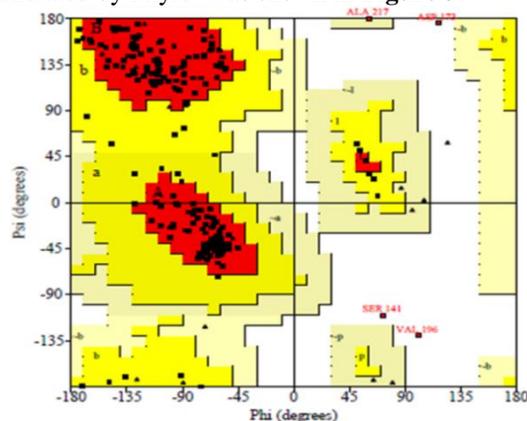


Fig. 3. Ramachandran plot of the cutinase model from *P. fluorescens* is determined by PROCHECK server. The most favored regions are marked as A, B, and L (red). The additional allowed regions are marked as a, b, l, and p (yellow), generously allowed regions are marked as ~a, ~b, ~l, and ~p (light yellow) and disallowed regions (white). All non-glycine and non-proline residues are shown as filled black squares, while glycines (non-end) are shown as filled black triangles.

PROCHECK validates refined 3D model by plotting the Ramachandran plot. Ramachandran plot statistics displayed that 223 amino acid residues (89.2%) are in the favored region, 23 amino acid residues (9.2%) are in the additional allowed region, and one amino acid residue (0.4%) are in the generously allowed region, while only three amino acid residues (1.2%) are in the disallowed region in cutinase. Hence, Ramachandran plot of the cutinase protein confirmed that the model structure is following dihedral angles of Ramachandran plot occupied favorable positions.

6. Functional analysis

Protein-protein interaction were performed using STRING 11.5 database, which is a biological database used to construct a protein-protein interaction network for different known and predicted protein interactions (SZKLARCZYK ET AL., 2019). Functional analysis revealed seven potential interacting partners of cutinase (node, B723_30130) from *P. fluorescens* in the protein interaction network as resolved by STRING 11.5 analysis with confidence scores 0.405-0.891. The closest interacting protein having the shortest node was found B723_30135 (AraC family transcriptional regulator) with the shortest node with a score of 0.891 while the distant interacting protein was found to be mraY (First step of the lipid cycle reactions in the biosynthesis of the cell wall peptidoglycan) and B723_08400 (Glycosyl transferase) with a score of 0.405 (Figure 4 and Table 5).

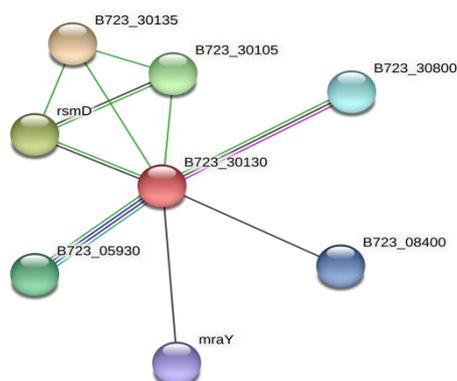


Fig. 4. Protein-protein interaction network for the cutinase of *P. fluorescens* detected through STRING database. The red node (cutinase from *P. fluorescens*) and other nodes represented its predicted functional partners from *P. fluorescens* (Table 5).

Table 5

Predicted functional protein partners of *P. fluorescens* cutinase

Node	Annotation
B723_05930	Glycerol acyltransferase
B723_08400	Glycosyl transferase
B723_30105	Peptidase M16
B723_30135	AraC family transcriptional regulator
B723_30800	ABC transporter ATP-binding protein
mraY	First step of the lipid cycle reactions in the biosynthesis of the cell wall peptidoglycan
rsmD	Specifically methylates the guanine in position 966 of 16S rRNA in the assembled 30S particle

The active site amino acid residues of cutinase, as determined using COFACTOR, were found to be leucine 69, serine 141, and leucine 142. BS-score is a measure of local similarity (sequence & structure) between template binding site and predicted binding site in the query

structure was 0.89 (> 0.5), representing a significant local match between the predicted and template binding site (**Figure 5**).

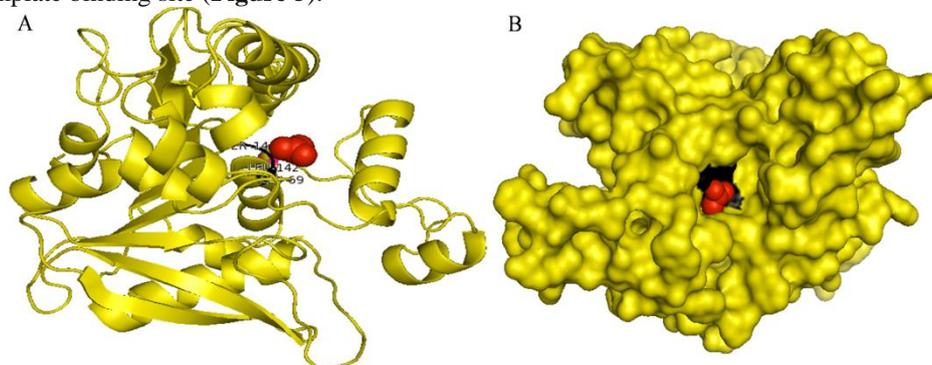


Fig. 5. Predicted ligand binding sites of cutinase from *P. fluorescens* visualized by PyMOL 2.3 molecular graphics software. (A) Showing three ligand binding sites (Leu 69, Ser 141, and Leu 142). (B) Surface view of the cutinase protein with ligand in pocket exposed active site. Spheres represent the ligand (red). Ligand binding sites (black) with in cutinase structure (yellow).

From the functional study of cutinase, four functional motifs were detected belonging to the α/β hydrolase superfamily, Serine aminopeptidase family, and cutinase family (**Figure 6**). Indeed, it has been shown that cutinase enzymes belong to the superfamily of α/β -hydrolase fold proteins with a conserved GX SXG motif and Ser-His-Asp catalytic triad (CHEN ET AL., 2008). In addition, our result is in concordance with several studies showing that unlike lipases, which have a secondary structure or hydrophobic “cap” that covers the active site, the most of cutinases do not have their catalytic esterase protected active site and that cutinases (MARTÍNEZ AND MAICAS, 2021) and that the α/β hydrolase superfamily have their catalytic serine exposed to the solvent along with a flexible active site (ZERVA ET AL., 2021). These enzymatic features can lead to a possible adaptation of the cutinase active site to different substrates and to large substrates such as cutin. This active site prediction is helpful in further docking analysis.

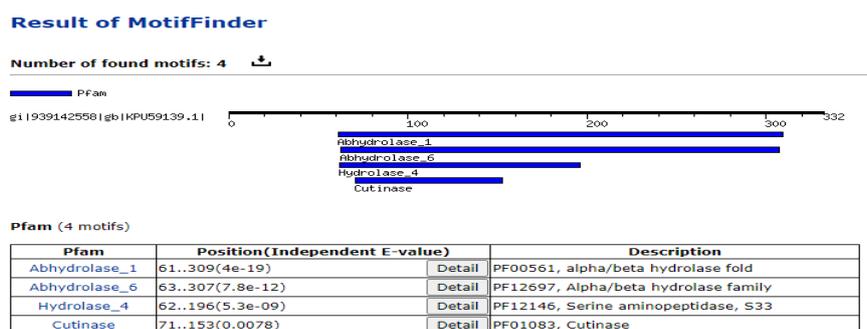


Fig. 6. Result of MOTIF finder showing functional motifs for the cutinase of *P. fluorescens*. Taken together, more structural and functional studies are needed to understand the structure and potential role in phytopathogenicity of the bacterial cutinase.

CONCLUSIONS

In recent years, cutinases from both phytopathogenic fungi and bacteria have been the center of attention. On the other hand, because of the non-availability of the crystal structures, *in silico*

analysis and homology modeling of protein structure is one of the very useful methods for studying the structure-function aspects of the protein of interest. This report can throw light into the cutinase protein structure, physicochemical properties, subcellular localization, structural and functional properties, and protein-protein interactions. Moreover, the present study underlines the potential in the prediction of enzymatic function, and of industrial and agronomic importance, PGPR and phytopathogenic bacterial cutinase enzyme characterization following relevant *in-silico* analyses.

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