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Identification and Molecular Characterisation of Pseudomonas florescence (ADY9 strain) as a Potential Bio-Pesticide

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Research Article

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ABSTRACT

ADY 9 strain was isolated from rhizospheric soil native to Uttarakhand's High altitude areas to test its efficacy as a promising biopesticide. The results indicate that the ADY 9 strain gives positive tests for catalase, chitinolytic activity, gelatine hydrolysis, carbohydrate utilization, protease, xylanase, beta-1,3glucanase. In addition to this it's effectiveness in managing grey spot disease in turnip was tested in vitro against with fungal inoculums. Outcomes of pot experiment showed the efficacy of formulation reduce disease incidence against control. Along with preventing pathological changes the formulation also induced some visible morphological changes such as increase in the root length, diameter and weight of the turnip in comparison with the control. For confirmation, we have done 16 sRNA sequences, polymerase chain reaction (PCR) amplification and nucleotide sequence analysis using BLAST (NCBI) as Pseudomonas florescence ADY 9 assigned the accession number MT549190 in GenBank. We display and define the genome sequence of Pseudomonas fluorescens strain ADY 9, which consists of a 1,391bp linear chromosome with GC material analysis, Neighbour Join, and a short minimum evolution graph. Furthermore, the ADY 9 strain's Swiss homology model has been explained in this paper.

INTRODUCTION

Fluorescent Pseudomonads belong to the PGPR (Plant Growth Promoting Rhizobacteria), the family of bacteria, which contribute to biocontrol, plant growth, process resistance, etc. Many varieties of Pseudomonas fluorescens are reported to promote plant growth and reduce the severity of various diseases. The effectiveness of antimicrobials in controlling fungal infections was often as good as on their own and sometimes combined with fungi. Pseudomonas fluorescens are aerobic, gram-negative organisms, present throughout the agricultural soil and extensively grown in the rhizosphere. This rhizobacterium possesses several characteristics that make it an effective biocontrol agent and aid in the growth of plants [1]. It is mass-produced and grows swiftly in the laboratory. It attaches and multiplies in both the rhizosphere and the spermosphere after rapidly using seeds and

root exudates. It generates a wide range of active metabolites in the rhizosphere, including volatile chemicals, siderophores, growth-promoting chemicals, antibiotics, and so on. Furthermore, pseudomonads are in charge of the natural suppression of harmful bacteria present in the soil. It inhibits pathogenic microbe development in several methods, including the generation of siderophores, other metabolites including phytoalexins and systemic resistance, hydrolytic enzymes such chitinase, and beta -1,3-glucanase, bacteriocins, and antibiotics [2]. This research paper mainly deals with isolation, identification, molecular characterization of ADY9 strain, testing its useful attributes as a promising biopesticide (field/pot trails).

MATERIALS AND METHODS

Collection of rhizospheric soil

Samples were collected from the rhizospheric soil of the higher region of Uttarakhand. Fluorescent Pseudomonas strain, isolated from the soil of Naugaon (26.348°N, 92.6838°E), Uttarakhand. Soil samples were taken from the rhizosphere of the rice plant up to a depth of 10 to 15 cm. To create a composite soil sample, the dirt that adhered to the roots was collected and combined.

Isolation of bacteria

The method was used to isolate Rhizobacteria. According to this, 10 g of soil was collected from each soil sample and placed in a conical flask holding 90 ml of distilled water. The mixture was vortexed for 15 minutes, then soil suspensions were diluted repeatedly. 0.1 ml of each dilution was placed on sterilised Petri plates with a specialised medium, such as Pseudomonas agar for fluorescein (Pseudomonas). The Petri plates were incubated at room temperature (28°C 2°C) for 24-72 hours. For each dilution, two duplicates were kept. Bacterial colonies were counted on the plates every day for up to three days. The plates were incubated for a day at 30°C for the formation of Pseudomonas colonies on KB plates, and the colonies were manually counted and documented shows the results Bacterial stock cultures were generated for storage at -20°C in 1.5 ml vials by combining equal amounts of 50% glycerol and 24-h culture broth (from a single colony inoculum, 25 ml NB medium, 100 ml flask, 130 rpm) and at 4°C in plates for daily routine work.

Morphological and biochemical analysis

The morphological and microscopically characterisation of isolated strains were observed by a statement of Bergey's Manual of Determinative Bacteriology; Gram Staining, Catalase Test, Motility Test, Starch Hydrolysis Test. Chitinolytic activity, gelatine hydrolysis, protease, beta-1,3glucanase, IMViC (Hi-media) and The capacity of the bacteria to thrive on carbon sources such as glucose, arabinose, lactose, Sorbitol, mannitol, rhamnose, sucrose, and xylose was examined using basal medium.

Preparation of a biopesticide inoculum

The ADY 6 strain was grown on King's B broth for 36 hours on a rotary shaker at 150 rpm to produce rhizobacterial inoculum for seed bacterization. Bacterial cells pelleted from the log phase were collected by centrifugation at 8000 rpm for 10 minutes and spectrophotometrically adjusted to 1 × 108 cfu/ml.

Cow urine and dung as a carrier material

Add 250 gm of cattle dung and 500 ml of cattle urine in a beaker and mix it well with help of a shaker. Cover the flask with aluminium foil and ferment at 37°C for 15 days and stir with the help of a shaker [3]. After 15 days, the solution was sieved with a sterile muslin cloth and adds bacterial suspension. The formulation was packed in a sterile container and stored at 4°C.

Seed Treatment and sampling

Turnip seeds were surface sterilised before being sowed in open furrows in the field under natural circumstances. The crop was grown by recommended agronomical procedures.
Soil samples were obtained from each plot before and after seeding at 0, 30, 60, and 90 days after sowing, as well as at harvesting. For this, 5 plants per plot were uprooted for the study of various crop growth parameters.

Molecular characterisation of ADY 9 strain

The CTAB technique was used to harvest DNA from overnight bacteria cultures. A set of 16S rRNA primers indicated as two universal primers GM3f (5' AGAGTTTGATCMTGGC 3') and GM4r (5' TACCTTGTTACGACTT 3') was used for PCR amplification. 1 × PCR buffer, 0.2 mMdNTPs mixes, 3 mM MgCl2, 1M forward and reverse primers, genomic DNA, 1U Taq Polymerase, and sterile distilled water were used in each PCR reaction mixture. The PCR reaction was set for 30 cycles on an Applied Biosystems PCR equipment (Verify 96 Well Thermal Cycler).

The following PCR profile was used: 5 minutes of initial denaturation at 95°C, 1 minute of denaturation at 94°C, 1 minute of annealing at 60°C, and 2 minutes of extension at 72°C. The last cycle comprised a 5-minute extension at 72°C to guarantee that the PCR products were fully extended. For 1 hour, 1 percent agarose gel electrophoresis was done at 80 V, 400 A. Finally, the PCR products were purified before they were sent out for sequencing. The collected raw sequencing data were then processed using the BLAST-N programme, which is accessible from the National Center for Biotechnology Information (NCBI). PhyIML software was used for sequence alignment and comparison.

G+C content analysis

Before assembly, the aforementioned contigs were BLAST-ed to discover similar sequences as well as terminal ends for each contig. SnapGene V.5.0.5 was used to construct the contigs. Snap gene programme was used to analyse GC content.

Swiss model homology analysis

SWISS-MODEL was used to accomplish automated homology modeling. The SWISS-MODEL server homology modeling process computes models based on ProMod3, an in-house comparison modeling engine based on Open Structure. A Swiss PDB viewer was used for interactive display and study of molecular structures. The SWISS-MODEL Workspace is a personal web-based working environment in which many modeling projects may be run concurrently. Protein sequence and structural databases, which are required for modeling, are available from the workspace and are updated regularly. Tools for template selection, model creation, and structure quality evaluation may be accessed directly from the workspace or via the web page menu.

RESULTS AND DISCUSSION

In this analysis, the ADY 9 strain was identified using morphological, biochemical and molecular criteria. The data on the morphological analysis included gram's negative, rod, single, round, smooth, convex colony, white and distinctively putrid odour colonies, while Gram staining and biochemical tests were described in (Table 1).

Biochemical analysis

SYSTEMIC Bacteriology, colony morphology was examined using three main features: shape, elevation, and margin. These core characteristics of bacteria colonies are an important criterion for colony recognition. When the ADY 9 strain was examined under a light microscope, it appeared as a rod [4]. Gram staining was used to identify various Gram bacteria. Gram-positive bacteria have a purple stain on their cell because of the dense peptidoglycan cell wall, while Gram-negative bacteria have a pink stain because of the thinner layer of the bacteria cell membrane. Gram staining analysis revealed that the ADY 9 bacterial strain produced Gram-negative results.

In the biochemical test, the strain tested positive for catalase because it was able to manufacture catalase enzyme, which allows it to catalyze hydrogen peroxide into oxygen and water. As a result, bubbles reflecting oxygen released by the breakdown of hydrogen peroxide generated during the catalase strain test. In the oxidase test, bacteria created cytochrome-c-oxidase, which catalyzes the reaction of cytochrome and oxygen, resulting in no production of the blue or deep purple color. Furthermore, the bacterial strain tested negative for indole. Bacterial strains were shown to be motile in the motility test (Table 2).

Table 1. Biochemical assay result of ADY 9 bacterial strain.

S. No.	Biochemical assay		Result	
			Positive	Negative
1	Gram Staining		-	Negative
2	Catalase		Positive	-
3	Indole		-	Negative
3	Motility		Positive	-
4	Methyl Red		-	Negative
5	Starch Hydrolysis		-	Negative
6	Voges-Proskauer		-	Negative
7	Chitinolytic activity		Positive	-
8	Gelatine hydrolysis		Positive	-
9	Protease		Positive	-
10	beta-1,3glucanase		Positive	-
11	Carbohydrate	Glucose	Positive	-
		Arabinose	-	Negative
		Lactose	Positive	-
		Sorbitol	-	Negative
		Mannitol	Positive	-
		Rhamnose	Positive	-
		Sucrose	Positive	-

Table 2: Percentage of disease incidence, root length, diameter of fruit, and weight of fruit in turnip plant treated with biopesticide.

Parameter	Days	Control	Treatment	SEm ±	C.D at 5%	CV (%)
Disease Incidence	D30	20.1	20.6	0.25	0.35	1.73
	D60	27.4	22.1	2.65	3.74	15.14
	D90	28.9	19.15	4.875	6.89	28.69
Root length	RL30	5	7	1	1.41	23.57
	RL60	7.4	9.4	1	1.41	16.83
	RL90	10.1	12	0.95	1.34	12.15
Diameter of fruit	DF30	13.3	19.1	2.9	4.1	25.31
	DF60	17.4	19.4	1	1.41	7.68
	DF90	19.1	21.5	1.2	1.69	8.35
Fruit weight	WF30	10	11.4	0.7	0.98	9.25
	WF60	14	20.56	3.28	4.63	26.84
	WF90	20	32	6	8.48	32.63

Biopesticide trail

Seeds treated with biopesticide formulations showed good growth responses across all field culture criteria, including disease incidence, root duration, fruit diameter, and fruit weight. Disease incidence increases in untreated control as 30 days, 60 days, and 90 days (11.5, 27.1, and 28.9) as compared with treated as 30 days, 60 days, and 90 days (14.1, 24.15, and 20.6). Soil analysis was changed in control and treatment. Statistis

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Analysis was done by GraphPad Prism 9.1.0 software. *In-vitro* analysis of Aspergillus brasiliensis was shown zone of Inhibition (Table 3) (Figures 1-3).

Table 3. Soil analysis of treated and untreated (Control) soil of turnip.

	Control				Treated			
	0 days	30 days	60 days	90 days	0 days	30 days	60 days	90 days
pH	7.14	7.26	7.45	7.46	7.25	7.37	7.55	7.6
Solubility	0.12	0.19	0.33	0.41	0.18	0.16	0.18	0.43
Carbon	0.65	1.05	0.63	0.71	0.96	1.09	1.1	1.98
Potash	225.3	231.9	224.9	232.1	229.1	24	262.7	270
Phosphorous	48.4	52.6	58.24	59.1	42.7	49.28	60.9	64.9
Sulphur	15.51	28.1	34.3	35.5	20.45	31.49	51.79	60.81
Zinc	0.68	1.41	4.72	4.8	1.14	2.19	4.79	5.11
Iron	15.32	23.89	21.38	23.81	21.98	24.6	29.88	38.43
Magnese	2.36	2.89	1.44	1.6	2.5	4.09	5.08	6.83
Copper	0.68	1.23	1.22	1.72	1.32	1.7	2.82	2.91

Figure 1: Graphical representation of parameter study in turnip field trail.

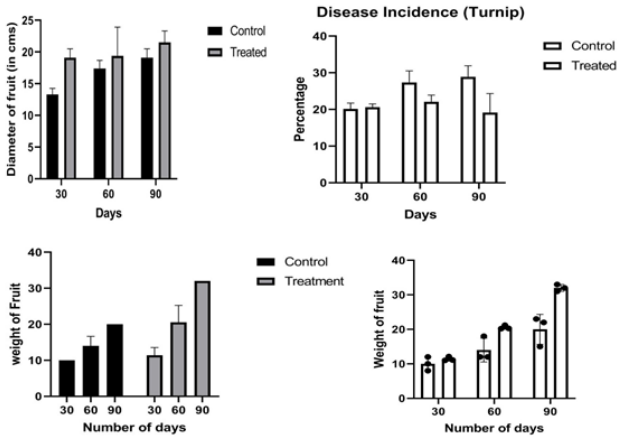

Figure 2: Graphical representation of physio-chemical properties of soil in turnip field trail.

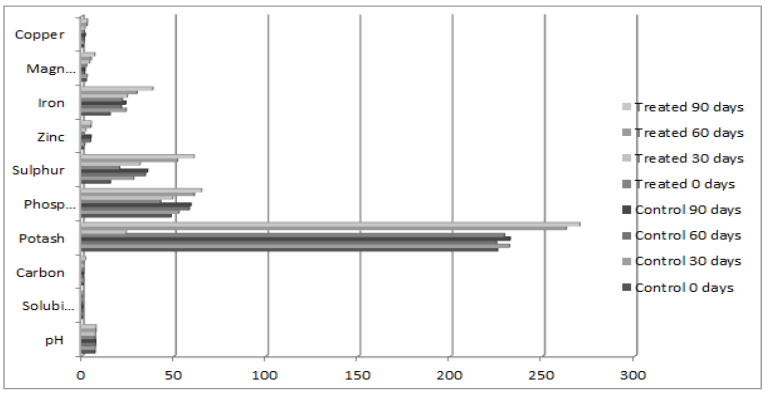

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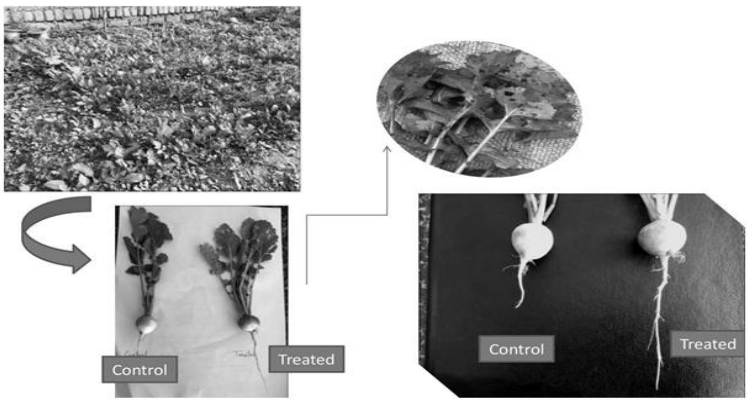
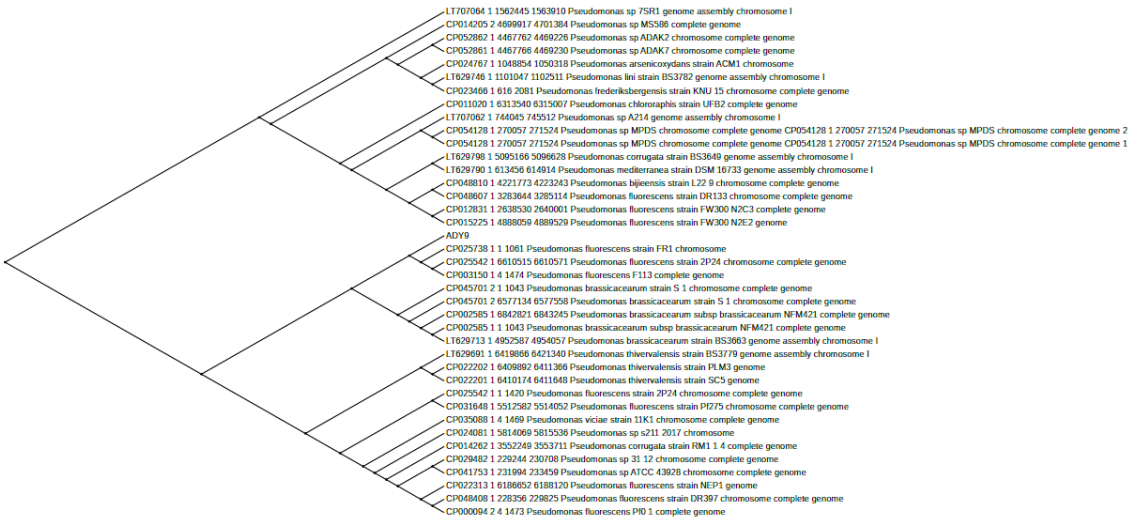
Figure 3: Field of turnip (gray-spot) disease in plant leaves.



Molecular identification

Amplification of the 16S rRNA gene was conducted for molecular characterisation of bacteria identification since this region gene sequences are usually present and universal for bacteria domains of multigene families. The 16S ribosomal gene has a length of 1492 base pairs, which is sufficient for genotypic verification. Using Gm3R and Gm3F primers, the PCR amplified products were 1492 bp in size. These universal primers were utilized to amplify a specific area of the 16S rRNA gene sequences that were assumed to be universal for the bacterium domain. The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST), and the results were compared to the existing sequences in the National Center for Biotechnology Information (NCBI) database, and the sequence of Pseudomonas fluorescence ADY 9 has been deposited in GenBank (Accession number- MT549190) [5]. The BLAST nucleotide analysis result and % similarity were displayed. The bulk of the isolated strains had a greater percentage similarity with 99.63 percent similarity and query coverage of 99 percent, according to the data. The accuracy of recognising unknown bacteria was established by utilising a percentage of similarity of 95 percent and 98.7 percent as a threshold value for genus and species taxonomy, respectively. iTol programme was used for phylogenetic analysis (Figure 4).

Figure 4: Phylogenetic expression of Nucleotide sequences.



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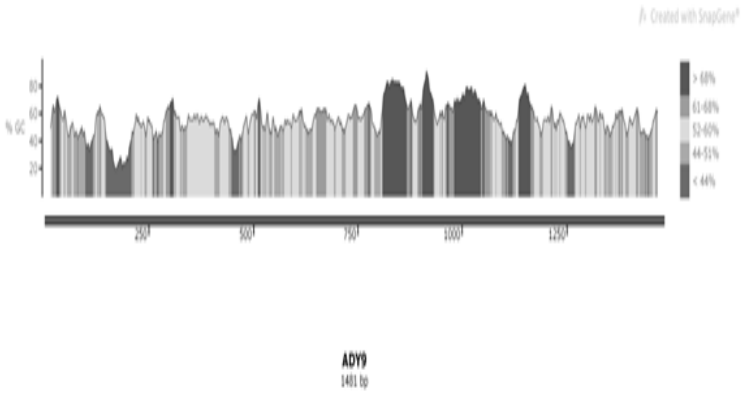
GC Percentage analysis

Snapgene Version 5.2 improves visualization and performance. In map view, a GC-content colour or line plot was displayed, and bases can be colored by GC content percentage (Figure 5).

Figure 5: % of GC- content of ADY 9.



Swiss-Model homology structure

Following the molecular characterization, we go on to the protein interaction between the strains, this can aid in future strain efficiency research. After the refinement step, the stereochemical quality of the predicted models and the validity of the protein model were evaluated using Ramachandran Map calculations generated by the Rampage program. The key chain metrics displayed include Ramachandran plot quality, peptide bond planarity, poor non-bonded contacts, main chain hydrogen bond energy, C-alpha chirality, and overall G factor. The Ramachandran plot analysis categorized the residues based on their quaternary structural positions. The residues were categorized using the Ramachandran plot analysis based on their quadrangle areas. The 3 Dimensional proteins created for Pseudomonas fluorescence ADY 9 were analyzed using Modeller software, and the findings indicated that the permissible areas of residues are 96.63, 93 percent, and so on. The distribution of main chain bond lengths and bond angles was discovered to be within the limitations of these proteins. The Ramachandran7 plot assigns such results to suggest that the projected models are of excellent quality (Figures 6 and 7) (Tables 4-6).

Figure 6: 1-11 Ramachandran plot of model of ADY 9.

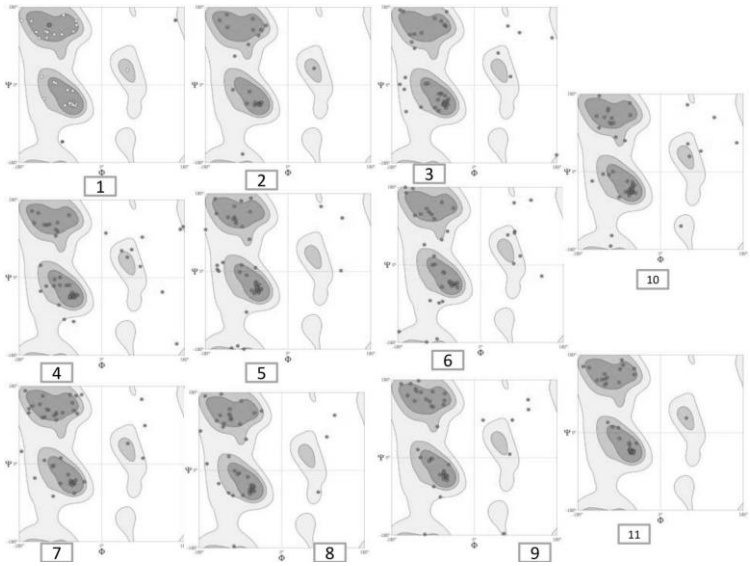


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Figure 7: Swiss-model 3-D protein structure, PPI fingerprint and Molecular interaction.

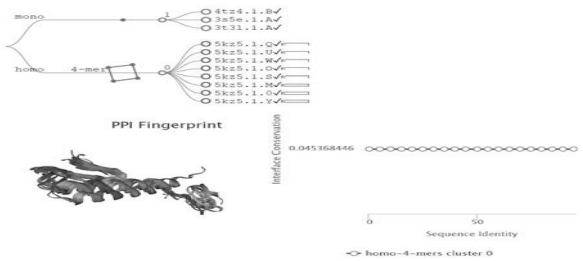

Table 4: Number of templates details through Swiss-homology model of ADY 9 strain.

Model	Template	Seq Identity	Oligostate	Seq Similarity	Coverage	Description
1	3s5e.1.A	12	monomer	0.29	0.1	Frataxin, mitochondrial
2	5kz5.1.M	10.42	homotetramer	0.29	0.1	Frataxin, mitochondrial
3	5kz5.1.O	10.42	homotetramer	0.29	0.1	Frataxin, mitochondrial
4	5kz5.1.Q	10.42	homotetramer	0.29	0.1	Frataxin, mitochondrial
5	5kz5.1.S	10.42	homotetramer	0.29	0.1	Frataxin, mitochondrial
6	5kz5.1.U	10.42	homotetramer	0.29	0.1	Frataxin, mitochondrial
7	5kz5.1.W	10.42	homotetramer	0.29	0.1	Frataxin, mitochondrial
8	5kz5.1.Y	10.42	homotetramer	0.29	0.1	Frataxin, mitochondrial
9	5kz5.1.0	10.42	homotetramer	0.29	0.1	Frataxin, mitochondrial
10	3t3l.1.A	10.42	monomer	0.28	0.1	Frataxin, mitochondrial
11	4tz4.1.B	12.5	monomer	0.3	0.07	Protein cereblon

Table 5: Quality estimation of different model.

Model	Quality estimation				
	QMEAN	Cβ	All atom	Solvation	Torsion
1	-1.82	-2.73	-1.04	-0.16	-1.48
2	-3.5	-2.69	-1.18	-2.61	-2.44
3	-5.5	-2.03	-2.56	-2.22	-4.89
4	-5.8	-2.62	-2.4	-1.63	-5.35
5	-6.88	-3.29	-3.19	-3.14	-5.73
6	-7.08	-3.51	-2.36	-2.26	-6.37
7	-5.05	-2.5	-2.65	-2.29	-4.16
8	-4.95	-3.04	-2.56	-2.86	-3.64
9	-5.75	-3.65	-2.79	-2.63	-4.5
10	-5.28	-3.47	-2.34	-2.5	-4.08
11	-1.15	-1.44	-0.51	-0.52	-0.76

Table 6: MolProbity of different model.

S.no	MolProbity							
	MolProbity Score	Clash Score	Ramachandran Favored	Ramachandran outliers	Rotamer Outliers	C-Beta Deviations	Cis Non-Proline	Twisted Non-Proline
1	2.08	14.3	93.62%	A46 HIS, A44 ILE (4.26%)	0.00 %	A44 ILE (1)		
2	1.13	0	86.67%	B80 VAL, B71 PRO (6.67%)	0.00 %	0		
3	2.19	6.8	72.55%	M15 VAL, M52 GLU, M54 LYS, M53 ILE, M14 PRO, M38 ILE (11.76%)		M52 GLU, M62 VAL, M12 MET, M13 ALA(4)	(M10 ALA-M11 GLY), (M11 GLY-M12 MET), (M12 MET-M13 ALA), (M21 PHE-M22 ARG), (M52 GLU-M53 ILE) (5/51)	
4	2.48	(045 HIS-046 HIS) (6.80)	70.59%	052 GLU, 053 ILE, 015 VAL, 042 ARG, 054 LYS, 046 HIS, 047 THR, 045 HIS (15.96%)	045 HIS-2.22 %	047 THR, 053 ILE, 055 LYS (3)	(010 ALA-011 GLY), (011 GLY-012 MET), (012 MET-013 ALA), (021 PHE-022 ARG), (052 GLU-053 ILE) (5/51)	(045 HIS-046 HIS) (1/51)
5	2.74	7.94	72.55%	Q14 PRO, Q15 VAL, Q12 MET, Q56 GLY, Q48 ALA, Q35 LEU (11.76%)	Q54 LYS, Q16 LEU (4.44 %)	Q40 ALA, Q23 LYS, Q10 ALA (3)	(Q10 ALA-Q11 GLY), (Q11 GLY-Q12 MET), (Q12 MET-Q13 ALA), (Q21 PHE-Q22 ARG) (4/51)	
6	2.53	7.94	70.59%	S53 ILE, S54 LYS, S14 PRO, S15 VAL, S38 ILE-9.80%	S49 LEU (2.22 %)	S21 PHE, S12 MET, S54 LYS, S53 ILE, S23 LYS (5)	(S10 ALA-S11 GLY), (S11 GLY-S12 MET), (S21 PHE-S22 ARG), (S52 GLU-S53 ILE) (4/51)	(S12 MET-S13 ALA) (1/51)
7	2.62	23.81	78.43%	U12 MET, U11 GLY, U52 GLU, U17 ARG, U53 ILE, U14 PRO (11.76%)		U52 GLU, U53 ILE, U35 LEU, U55 LYS, U21 PHE (5)	(U10 ALA-U11 GLY), (U11 GLY-U12 MET), (U12 MET-U13 ALA), (U21 PHE-U22 ARG), (U47 THR-U48 ALA), (U52 GLU-U53 ILE) (6/51)	
8	2.75	(W45 HIS-W49 LEU) 17.01	78.43%	W12 MET, W56 GLY, W53 ILE, W35 LEU (7.84%)	W16 LEU (2.22 %)	W53 ILE, W40 ALA, W51 ILE (3)	(W10 ALA-W11 GLY), (W11 GLY-W12 MET), (W12 MET-W13 ALA), (W21 PHE-W22 ARG), (W52 GLU-W53 ILE) (5/51)	
9	2.78	18.14	78.43%	Y14 PRO, Y52 GLU, Y23 LYS, Y13 ALA, Y35 LEU, Y54 LYS, Y53 ILE (13.73%)	Y45 HIS (2.22 %)	Y45 HIS, Y53 ILE, Y13 ALA, Y40 ALA, Y54 LYS, Y52 GLU, Y23 LYS, Y43 ARG (8)	(Y10 ALA-Y11 GLY), (Y11 GLY-Y12 MET), (Y12 MET-Y13 ALA), (Y21 PHE-Y22 ARG), (Y47 THR-Y48 ALA), (Y52 GLU-Y53 ILE) (6/51)	
10	2.68	13.61	76.47%	015 VAL, 012 MET, 017 ARG, 035 LEU, 014	016 LEU (2.22	053 ILE, 015 VAL, 052 GLU, 014	(010 ALA-011 GLY), (011 GLY-012 MET), (012 MET-013 ALA), (021 PHE-022	(013 ALA-014

				PRO, 053 ILE (11.76%)	%)	PRO, 013 ALA, 040 ALA, 017 ARG(7)	ARG), (052 GLU-053 ILE) (5/51)	PRO) (1/1)
11	1.85	(A45 HIS-A49 LEU) (11.14)	100 %		A16 LEU (2.38 %)	A16 LEU (1)		

CONCLUSION

The bacteria isolated were known as Pseudomonas fluorescence ADY 6 strain, and the sequence was sent to NCBI (Assession number-MT549190). Biopesticide formulations were successful against grey spot disease. The 3D model of predicted protein interactions was developed in this study using homology modelling with SWISS-MODEL. Molprobity, RAMPAGE, and Zscore were used to further test the final refined model. The predicted 3D structure would aid in the interpretation of Pseudomonas fluorescence ADY 6 protein structure.

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CONFLICTS OF INTEREST

Non conflicts of interest

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