

Studies on the Effects of L-Prolinamide, 5-OXO-L-Prolyl-L-Phenylanyl-4-Hydroxy Compound Produced by Pseudomonas Fluorescence against Cell Wall Protein (3GNU Receptor) of Pythium SPP MTCC 10247

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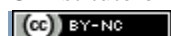
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ABSTRACT

The present study was carried out in order to assess the biocontrol efficiency of *Pseudomonas fluorescence* against *Pythium* spp MTCC 10247. The earlier studies have revealed its antifungal properties but the previous reports lack the details about the compounds and the target for the fungi. Hence we have designed our work plan to reveal the compounds present in the secondary metabolites of *Pseudomonas fluorescence* and the target on the phytopathogens *Pythium* spp MTCC 10247 which causes plant diseases. Production of secondary metabolites of *Pseudomonas fluorescence* was carried out and the compounds were characterized by TLC, SDS-PAGE, and GC-MS. There were 7 peak compounds found out, they are 1,4-diaza-2,5-dioxobicyclo[4.3.0] nonane (13.69%), 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione(10.11%), Pyrrolo[1,2-a] pyrazine 1,4dione,hexahydro-3-(2-methylpropyl)(17.79%) l-Leucine,N-cyclopylcarbonyl-pentadecyl ester(7.09%), 3,6diisobutyl-2,5-piperazinedione (37.62%), 3-benzylhexahydropyrrolo[1,2-A]pyrazine-1,4-dione(8.52%), L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4-hydroxy(5.19%). Antifungal activity of the ethyl acetate extract of the secondary metabolites were examined against *Pythium* spp MTCC 10247 and result revealed that the lag period of the fungi was double fold higher than the control and the compounds were fungi static because there was a 57% of growth reduction after 7th day of incubation period. All the seven compounds were used for molecular docking study and result showed higher score value with L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4-hydroxy against cell wall protein (3GNU receptor) *Pythium* spp MTCC 10247.

Key Words: *Pseudomonas fluorescence*, *Pythium* spp MTCC No 10247, GC-MS, Anti-fungal activity, molecular docking, L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4-hydroxy, 3GNU.

Introduction:

Paddy soil Bacteria which are have potential to control destructive diseases are distributed in many genera. Among them *Pseudomonas fluorescence* are most effective bacteria for bio control of soil and foliar disease. *Pseudomonas fluorescence* which have various modes of action especially antibiotic production, rhizosphere colonization etc., would certainly be potential bio control agents for the management of pest and crop plant diseases [1, 2].

More than 10,000 species of fungi which can cause disease in paddy plants. most commonly cause diseases in agricultural crops are Plasmodiophoromycetes (cause club root of crucifers, root disease of cereals and powdery scab of potato), Oomycetes (cause seedling damping-off, late blight, downy mildews, and white rust disease), Zygomycetes (cause soft rot of fruit), Ascomycetes and Deuteromycetes (cause leaf spots, blights, cankers, fruit spots, fruit rots, anthracnoses, stem rots, root rots, vascular wilts, soft rot), and Basidiomycetes (cause rust and smut diseases [3,4].

Technological improvement, which have more effective use of natural resources, is required in agriculture. Many microbial antagonists have antagonistic activities against plant fungal pathogens, such as *Pseudomonas fluorescens*, *Agrobacterium radiobacter*, *Bacillus subtilis*, *B. cereus*, *B. amyloliquefaciens*, *Trichoderma viride*, *Trichoderma harzianum*, *Burkholderia cepacia*, *Saccharomyces* spp and *Gliocadium virens* [5, 6, 7].

Some of bacteria, fungal pathogens causes serious damage in agriculture, finally losses of yield, quality and profit. Chemical insecticides are normally used against

phytopathogens. Due to serious demerits of using chemical insecticides, bio insecticides or bio pesticides are used as Alternatives, enhance crop yield by growth promoting attributes of environment friendly microbes, this method has been developed successfully during the last few years. Many organism which are shown to have pathogenic activity naturally .secondary metabolites produced by *Pseudomonas fluorescence* showed bio control activity against the soil borne Phytopathogenic fungi and has the potential to produce known secondary metabolites such as siderophore, antibiotic, voltaic compound ,HCN ,Protease and phytohormones that showed the antagonistic activity against *Macrophomina phaseolina*, *Rhizoctonia solani*, *Phytophthora nicotianae* var. *parasitica*, *Pythium* sp. and *Fusarium* spp [8,9].

Materials & Methods:

Collection of Sample:

The commercially available bio pesticide *Pseudomonas fluorescence* was collected from the farmers of Wayanadu, Kerala and which was used for the further study to find out the similar biocontrol efficiency of the bacteria against the predominant pests infesting the crops of Tiruporur agriculture area of Tamil Nadu

Isolation of *Pseudomonas fluorescence*:

Pseudomonas fluorescence biopesticide powder was collected from Wayanadu, kerala was further reviewed using nutrient agar and King's B medium[10].

Production of Biomass & Secondary Metabolites

Pseudomonas fluorescence isolated using the solid media was inoculated in two conical flasks containing 100 ml of sterile Nutrient broth and two conical flasks containing 100ml of sterile King's B broth. The media were incubated at 37°C and Biomass was collected after 48 hours by centrifugation at 10,000rpm for 30 minutes. Secondary metabolite extraction was done after 72 hours of incubation period [11].

Extraction of Secondary Metabolites

The 72 hours old culture was used for this extraction. Culture was centrifuged at 10,000 rpm for 30 minutes and the supernatant was collected. Solvent extraction procedure was carried out using ethyl acetate (1:1). Middle layer was collected and samples were air dried and used for further characterization [12].

Extraction of Secondary Metabolites by Ethyl Acetate Solvent Extraction Method

The K'B broth was centrifuged and the 40ml of supernatant was separated.50 ml of ethyl acetate and 50ml of supernatant are transfer in separating funnel and is mixed for 30mins.Then the funnel was kept in stand at room temperature for three hours. After 3 hours 3 layers were separated out. The three layers were carefully collected out. The middle layer was used for further analysis [13].

Thin Layer Chromatography

The ethyl acetate extract samples were applied on the bottom of the activated TLC plate using capillary tubes. It was then kept in TLC tank containing the mixture of chloroform: ethyl acetate: acetic acid in the ratio of 100:80:20 ml .The top of the TLC tank was closed with a thick glass plate to avoid the evaporation of solvents. The mobile phase moved up by capillary action and thus the active constituents were separated based on their solubility. The TLC plate was take out when the solvent front reaches the top of the plate. Then the 2% ferric chloride solution was sprayed on plate and dried in 10 minutes in hot air oven. Then the TLC plate viewed under UV

light. All the sample have fluorescent nature, thus fluoresced under UV light. The TLC plates showed fluorescent bands/compounds.

Thin Layer Chromatography: Amino Acid

Thin layer chromatography was used to check the active constituents present in *Pseudomonas fluorescense* (ethyl acetate)

SDS PAGE [Sodium Dodicysulphate Polyacrylamide Gel Electrophoresis]:

10% Stock acrylamide-13.3ml, Tris HCL-8ml [ph-8.8], Water-18.1ml, APS-200µl, 10%SDS-400µl, TEMED-20µl. Mixed well and poured the gel solution into the chamber and distilled water was added to top of the gel and left for 30 minutes.

4 % Stock acrylamide-1.35ml, Tris HCL-1ml [ph-6.8], Water-7.5ml, APS-50µl, 10%SDS-100µl, TEMED-10µl. the stacking gel mixture was poured into top of the chamber and comb was placed and allowed the gel to set 30mins. After stacking polymerized, the comb was removed carefully without damaging the wells. Clip was removed and bottom site of the spacers was removed. Electrode buffer was filled with electrophoresis apparatus. The gel plate inserted in to the electrophoresis apparatus.

A 200µl of sample buffer and sample was mixed well and heated it for 2 minutes, cool it, 50µl of sample was loaded in to the each well and run the sample.

After electrophoresis the chamber was removed from the electrophoresis apparatus. The side spaces were removed and gel was removed carefully from the chamber. The gel was transferred in to clean plastic container. Then the gel was kept inside the staining solution comassive blue solution overnight. Stain was discarded and detainer was done.

GC-MS (Gas Chromatography and Mass Spectrometry):

A 200µl sample was taken in beaker and ethyl acetate was added to it and is mixed by pipetting. Sample mixer was taken in syringe, 0.2 PTFE FILTER was inserted in syringe and test samples were filtered in 1.5ml of vial tube. In 4ml vial tube ethyl acetate was added. This 2 vial tubes were kept in injector, in injector the samples was washed and 1µl sample was injected in to inlet. After 43minutes the result peaks was observed in monitor.

Antifungal Activity:

PDA was prepared and sterilized at 121⁰c for 15 minutes. After sterilization the media was poured in Petriplates plate marked as positive control and control. In other plate 10ml media and 200µl compounds are mixed well and poured it in the plates it was marked as test, allowed to solidify. Then the loop of *Pythium spp MTCC 10247* culture was inoculated in test plate and positive control plate in the centre of plate. The plate was sealed and kept it in room temperature [14, 15].

Molecular Docking:

Molegro Virtual Docker is an integrated platform for predicting protein - ligand interactions (GC-MS 7 Major compounds and 3GNU *Pythium spp MTCC 10247*). Molegro Virtual Docker handles all aspects of the docking process from preparation of the molecules to determination of the potential binding sites of the target protein, and prediction of the binding modes of the ligands. The Molegro Virtual Docker (MVD) has been shown to yield higher docking accuracy than other state-of-the-art docking product [16].

Results and Discussion:

Pseudomonas fluorescense isolated by streak plate (King's B Media) method was found to be yellowish pigment on King's B revealed by UV Trans illuminator (fig 1). The fluorescing colonies were identified by phenotypic methods of The Bergy's Manual of systemic bacteriology (table 1). The results were shown in the table 1. Secondary metabolites of *Pseudomonas fluorescense* produced by 48 hours King's B broth, metabolic compounds were characterized from solvent extraction (ethyl acetate) method, Phenolic compounds were identified by Thin Layer Chromatography. The Gas chromatography and Mass Spectrometry of *P.fluorescense* ethyl acetate crude extract shows the appearance of the seven major compounds at different retention times. The results were shown in (table 2).

Isolated crude extracted *Pseudomonas fluorescense* were evaluated for antifungal activity against *Pythium spp MTCC 10247*. Mycelium growth of the *Pythium spp MTCC 10247* were reduced after the treatment with *Pseudomonas fluorescense* crude extracted in comparison with the control. The results were shown in the (table 3). The growth behaviour of the test fungi *Pythium spp MTCC 10247* with crude extract of antagonistic bacteria *Pseudomonas fluorescense* in 2nd day incubation period no growth in test plate and positive control plate (100% incubation of mycelium growth of *Pythium spp MTCC 10247*) , 3rd day incubation period no growth in test plate , 0.3 cm mycelium growth in positive control plate(100% incubation of mycelium growth of *Pythium spp MTCC 10247*), 4th day incubation period no growth in test plate , 3 cm mycelium growth in positive control plate(100% incubation of mycelium growth of *Pythium spp MTCC 10247*), 5th day incubation period no growth in test plate , 5 cm mycelium growth in positive control plate(100% incubation of mycelium growth of *Pythium spp MTCC 10247*), 6th day incubation period 1 cm mycelium growth in test plate , 7 cm mycelium growth in positive control plate(86% incubation of mycelium growth of *Pythium spp MTCC 10247*), 7th day incubation period 3 cm mycelium growth in test plate , 7 cm mycelium growth in positive control plate(57% incubation of mycelium growth of *Pythium spp MTCC 10247*) (fig 2) .

In molecular docking the antibiotics of 7 ligands of *Pseudomonas fluorescense* obtained from GC-MS result is dock on 3GNU receptor of *Pythium spp* (Table 4). Docking results revealed the docking score of L-Prolinamide,5-Oxo-L-Prolyl-L-Phenylalanyhydroxy(C19H24N4O5) on -147.511 and they had formed -13.795 hydrogen bond with the receptor(table 5) .based on the docking score results among the 7 compound this compound have higher binding affinity with the cell wall of *Pythium spp* (3GNU receptor) therefore the fungi shows sensitivity to *Pseudomonas fluorescense* of this compound L-Prolinamide,5-Oxo-L-Prolyl-L-Phenylalanyl-4-Hydroxy(fig 3,4,5,6,7).

The antifungal activity of *P.fluorescense* had reported against the *P.oryzae* and *R.solani* (17) *Pseudomonas fluorescense* was shown to effectively inhibit *P.oryzae* and *R.solani* by agar plating method reported that *P.fluorescense* inhibit a maximum of (50-85%) growth of 2 pathogens when compare to the control. The antifungal activity of *P.fluorescense* R21, F113 had reported against the *Pythium spp* (18) *P. fluorescense* metabolites 2, 4-diacetylphloroglucinol (DAPG). was shown to effectively inhibit *Pythium spp* By agar plating method reported that *P.fluorescense* R21 effectively inhibit a maximum of (58.3%) growth of *Pythium spp* and *P.fluorescense* F113 effectively inhibit a maximum of (66.7%) growth of *Pythium spp* when compare to control. In ours *Pseudomonas fluorescense* was effectively inhibit (5cm) the mycelial growth *Pythium spp*.

The antifungal activity of *Pseudomonas fluorescence* EBC5, EBC6, EBC7 had reported that *P. fluorescence* against the *Pythium aphanidermatum*(19) *P. fluorescence* EBC 5, EBC 7 and EBC 6 recorded that inhibit a minimum mycelial growth (28.00, 30.66 and 33.33 mm, respectively) with maximum inhibition zone of (12.33, 11.66 and 11.08 mm, respectively) of *Pythium aphanidermatum* over control. In our studies *Pseudomonas fluorescence* was effectively inhibit (5cm) the mycelial growth *Pythium spp*. The *in vitro* action of *P. fluorescens* against *P. aphanidermatum* obvious by the inhibition zones shows fungal activity of the bacterium. (20) Most authors showed antimicrobial activity of bacteria, particularly *Pseudomonas* species on fungal pathogens. Antifungal metabolite of *Pseudomonas antimicrobial* inhibited efficiently showed the establishment of inhibition zone due to *in vitro* antifungal activity of. Microscopic observations of mycelium tissue inside inhibition zone showed the absence of spore and the denaturation of the mycelium. The culture of a portion of this zone on the new PDA could not allow the mycelium development. This result could be suggested that compounds released by the bacterium are fungicides.

This result was also suggested hypothesis depending that *P. fluorescence* produced antibiotics responsible of the bio protection. Indeed, it was isolated more secondary substances produce by *P. fluorescence* which were fungicides antioomycete properties. In our studies *Pseudomonas fluorescence* was effectively inhibit (5cm) the mycelial growth *Pythium spp*. *Pseudomonas fluorescence* was effectively inhibit (57%) the mycelial growth of *Pythium spp*(Table 3).

Investigation on microbial metabolites is gaining greater momentum in the agrochemical industry as a source for the development of new pesticide products. Several such products have been developed and used as insecticide, bactericide, fungicide, acaricide, insecticide or acaricide in agriculture. However, research on new metabolites and its development, as agrochemical by fermentation technology has not been addressed adequately in India. Therefore, it is considered worthwhile to generate reliable data on the isolation, production, productivity and bio efficacy of antifungal metabolites from some native *P. fluorescence*. The metabolite(L-prolinamide,5-oxo-1-prolyl-1-phenylanyl-4-hydroxy) was isolated .the metabolite were extracted from *P. fluorescence* using ethyl acetate and studied bio control efficiency of solvent against the *Pythium spp MTCC 10247*, the metabolite extracted with ethyl acetate was effectively inhibited(60%).and our docking studies *Pseudomonas fluorescence* metabolite(L-prolinamide,5-oxo-1-prolyl-1-phenylanyl-4-hydroxy) which dock the 3GNU *Pythium* cell wall protein which confirm the presence antifungal compound in *P. fluorescence* it inhibiting the *Pythium spp MTCC10247* growth and is all so kill the paddy rice leaf folder pest (*Cnaphalocrocis medinalis*) comparing to others this investigation shows better control when compare to the control.

Conclusions:

Beneficial paddy soil bacteria *Pseudomonas fluorescence* was able to produce maximum concentration L-prolinamide, 5-oxo-1-prolyl-1-phenylanyl-4-hydroxy which possess antifungal activity against *Pythium spp MTCC 10247*, which can be extended with various phytopathogens for further application. . The presented data exhibit the antifungal activity of *Pseudomonas fluorescence* and indicate the possibility of using *Pseudomonas fluorescence* as a biological control agent of some plant pathogenic fungi. However, this requires further screening of a large number of *Pseudomonas fluorescence* from different regions of India. The antimicrobial activity of *Pseudomonas fluorescence* may be attributed to the various phytochemical constituents have even more potency with respect to the inhibition of microbes.

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(Tables & Figures)

Table 1: Showing Biochemical characterization of *Pseudomonas fluorescense*.

S.NO	NAME OF TEST	RESULT
1.	Gram's staining	Gram negative
2.	Motility test	Motile
3.	Flourescence under UV	Positive
4.	Indole test	Negative
5.	Methyl red test	Negative
6.	Vp test	Negative
7.	Gelatin plate test	Positive
8.	Gelatin tube test	Positive
9.	Nitrate test	Positive

Table 2: Showing Gas chromatography and Mass Spectrometry of *P.fluorescense* seven major compounds at different retention times.

Peak #	R.Time	Area	Area %	Compound Names
1	21.869	1723219	13.69	1,4-diaza-2,5-dioxobicyclo[4.3.0] nonane
2	23.704	1272404	10.11	3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione
3	25.684	2239437	17.79	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)
4	25.765	892870	7.09	1-Leucine,N-cyclopylcarbonyl-pentadecyl ester
5	29.961	4734481	37.61	3,6-diisobutyl-2,5-piperazinedione
6	32.797	1072542	8.52	3-benzylhexahydropyrrolo[1,2-A]pyrazine-1,4-dione
7	37.452	653379	5.19	L-prolinamide,5-oxo-1-prolyl-1-phenylanyl-4-hydroxy
		12588332	100.00	

Table 3: Showing inhibition of *Pseudomonas fluorescense* on the mycelium growth of *Pythium spp MTCC 10247*.

S.NO	INCUBATION PERIOD	TEST PLATE(cm)	POSITIVE CONTROL PLATE (cm)	% inhibition	NEGATIVE CONTROL PLATE
1.	2 nd day	No growth	No growth	-	No growth
2.	3 rd day	No growth	0.3 cm	100%	No growth
3.	4 th day	No growth	3 cm	100%	No growth
4.	5 th day	No growth	5 cm	100%	No growth
5.	6 th day	1 cm	7cm	86%	No growth
6.	7 th day	3cm	7cm	57%	No growth

Table 4: Showing docking score of 7 GC-MS *Pseudomonas fluorescence* compounds and *Pythium spp* cell wall 3GNU receptor.

Molecular name	Ligand	Moldock score	Re rank score	H BONDS
1,4-diaza-2,5-dioxobicyclo[4.3.0] nonane	45588	-72.007	-62.7619	-7.54215
3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	118054	-89.4117	-77.7031	-2.71803
Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)	50619	-89.4405	-75.7725	-3.54399
l-Leucine,N-cyclopylcarbonyl-pentadecyl ester	185528	-113.24	-39.8791	-3.55647
3,6-diisobutyl-2,5-piperazinedione	141382	-88.6453	-69.5163	-4.68542
3-benzylhexahydropyrrolo[1,2-A]pyrazine-1,4-dione	166293	-98.704	-31.7975	-9.35383
L-prolinamide,5-oxo-l-prolyl-l-phenylanyl-4-hydroxy	326452	-147.511	-104.321	-13.795

Table 5 : Showing the best docking score of L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4-Hydroxy (326452) and 3GNU receptor.

Ligand	MolDockScore	Rerank Score	H Bond
326452	-147.511	-104.231	-13.795

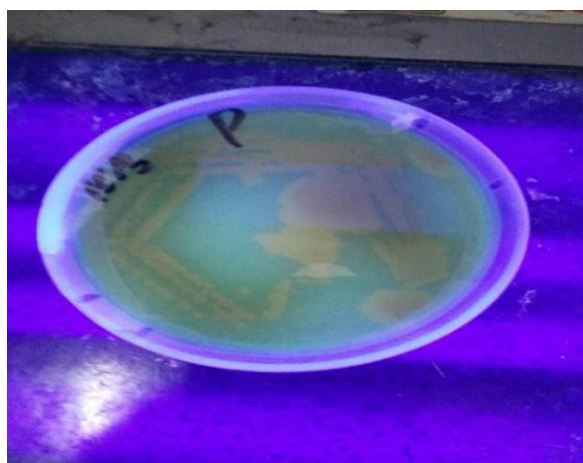


Fig 1: King's B plates shows fluorescence yellow colour colonies and indicates the growth of *Pseudomonas fluorescence*.

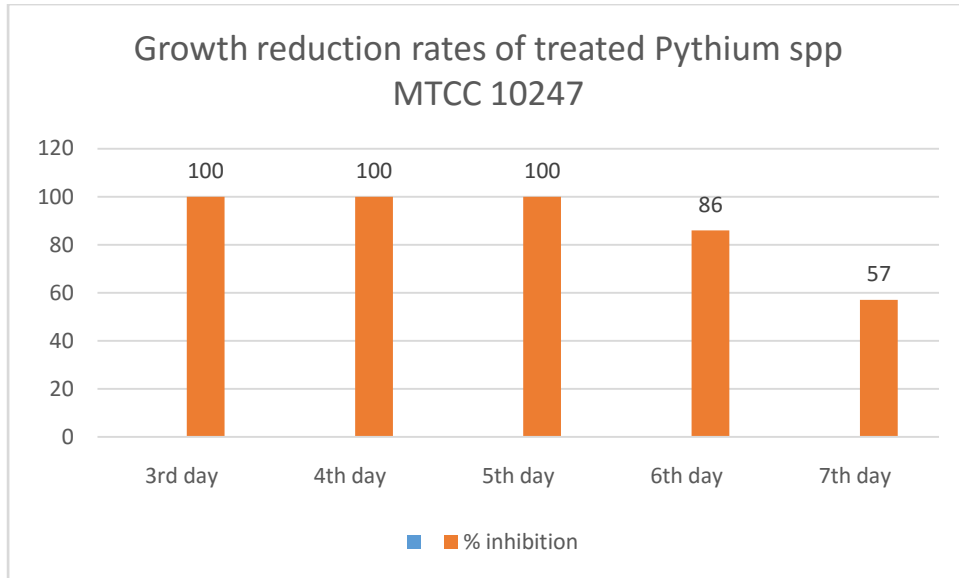


Fig 2: Showing inhibition of *Pseudomonas fluorescense* on the mycelium growth Of *Pythium spp* MTCC 10247

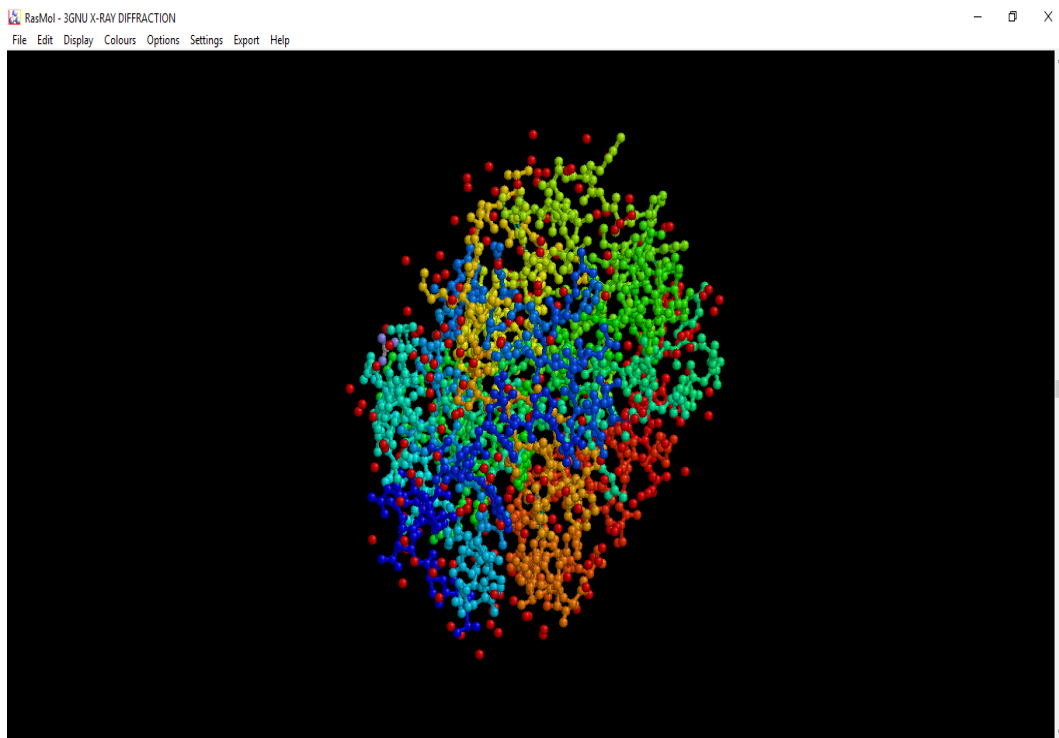


Fig 3: Showing 3D structure of 3GNU receptor

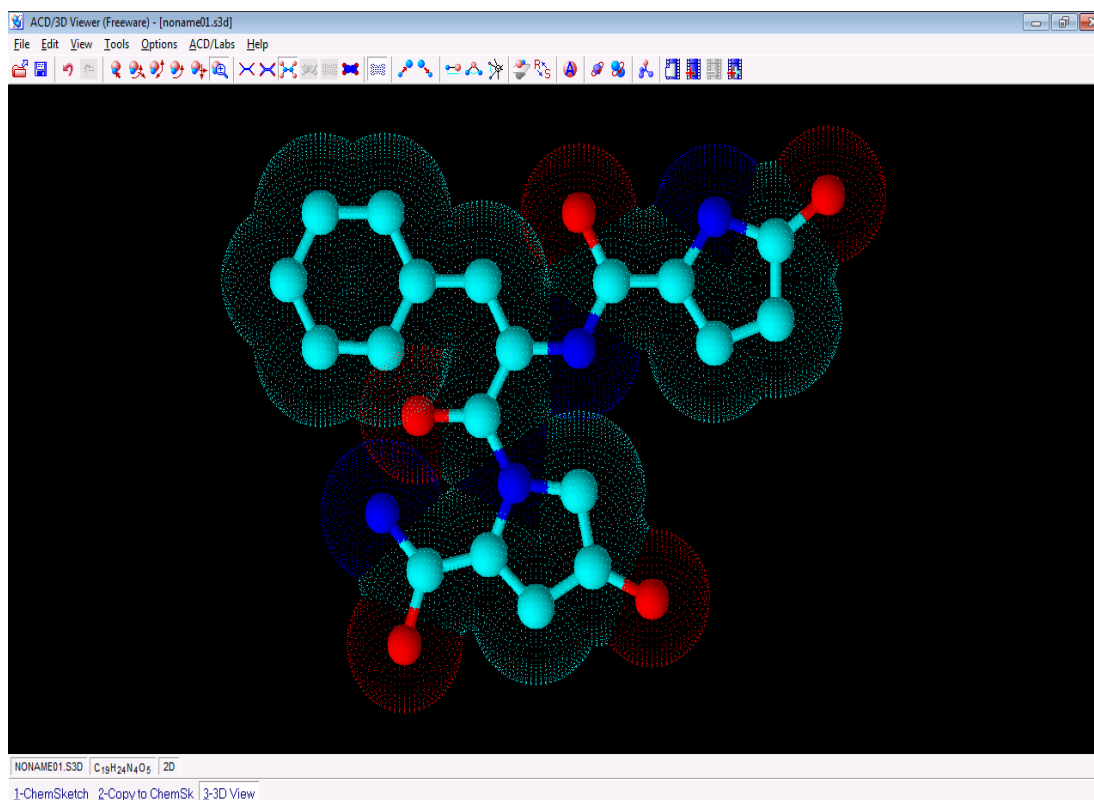


Fig 4: showing 3D Structure of L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4-hydroxy ligand.

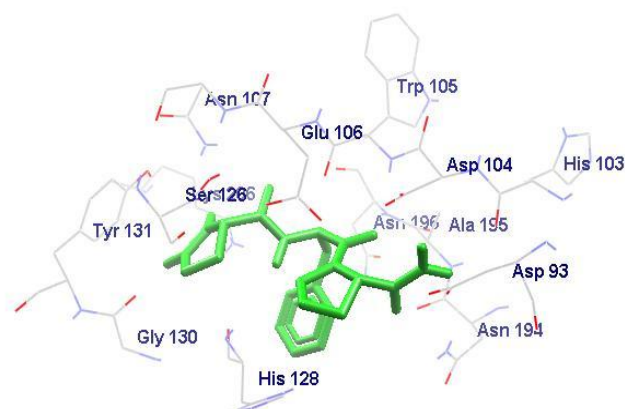


Fig 5: Showing L-Prolinamide, 5-Oxo-L-Prolyl-L-Phenylalanyl-4-Hydroxy (326452) ligand against 3GNU receptor /ligand pose (C19H24N4O5).

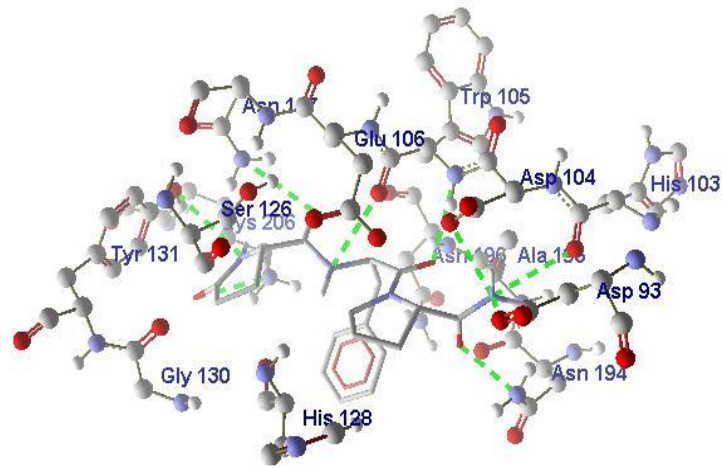


Fig 6: Showing active binding site of L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4-hydroxy (326452) and 3GNU receptor/ H Bond (Protein present on the surface of *Pythium spp MTCC 10247* cell wall has been highlighted in green color).

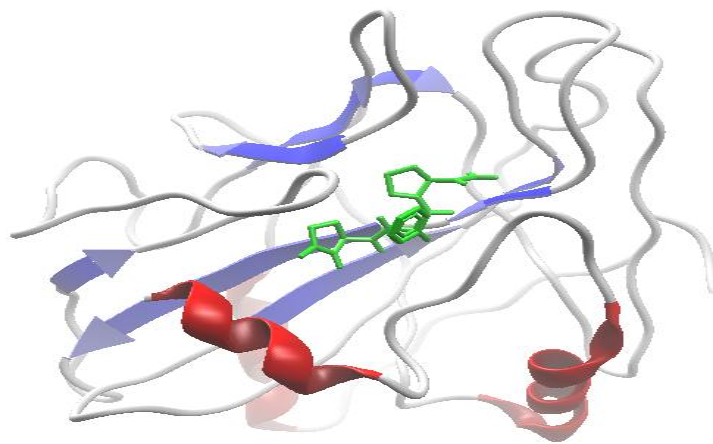


Fig 7: Showing secondary structure L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4-hydroxy (326452) and 3GNU receptor.

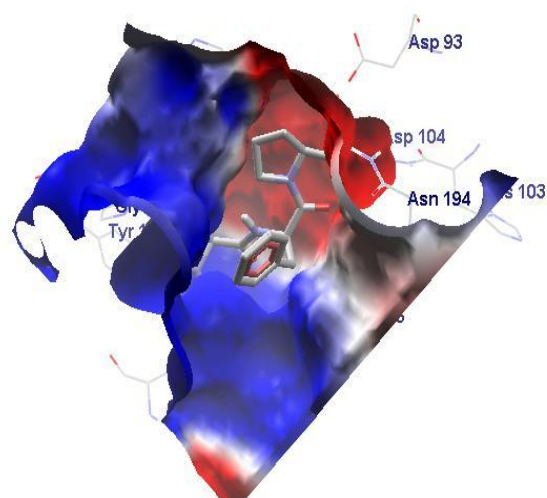


Fig 8: Showing Electrostatic Interaction between L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4- Hydroxy (326452) and 3GNU receptor.