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ACTIVITY OF METABOLIC ENZYMES TOWARDS EXHIBITING INSECTICIDE RESISTANCE IN LEPIDOPTERAN INSECT PEST: AN *IN-VITRO* AND *IN-SILICO* STUDIES

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Abstract

Insecticides are playing as pivotal role in agriculture worldwide towards protecting the crops from insect pests, their efficacy has been limited by the development of resistance in many major pests, including some that became pests only as a result of chronic and unscientific insecticide practice. Insecticide resistance is both an interesting example of the adaptability of insect pests, and also in the design of resistance management programmes. Resistance may be demarcated as 'a heritable change in the sensitivity of a pest that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the labeled recommendation for that pest species. This current study throws a light in understanding the mechanism of Insecticide resistance by considering 2 lepidopteran insect pests namely-*Helicoverpa armigera* and *Plutella xylostella* treated with organophosphate chemical, Dichlorvos collected from Dodaballapura of Bangalore rural district. The enzymatic activity of metabolic enzymes responsible for the exhibition of the resistance mechanism showed the escalation in Glutothione S transferase activity compared with Esterase activity in the field collected populations which created an curiosity to understand the metabolic enzymes at structural level using In-silico molecular docking using Autodock 4.0.

Keywords **:** Insecticideresistance, Lepidoptera, Insect pest, *Helicoverpa armigera, Plutella xylostella*, In-silico docking, Auto dock.

Introduction

The Lepidoptera order insects *Helicoverpa armigera* and *Plutella xylostella* is a destructive pests and causes enormous loss to global cruciferous crops. (Li *et al.,* 2016), (Juric *et al.,* 2017) The diamondback moth (DBM) *Plutella xylostella* is one of the most studied insect pests in the world, yet it is among the 'leaders' of the most difficult pests to control. It was the first crop insect reported to develop resistance to microbial *Bacillus thuringiensis* insecticides, and has shown resistance to almost every insecticide, including the most recent groups such as diamide. DBM is a highly invasive species. It may have its origin in Europe, South Africa or East Asia, but is now present wherever its cruciferous hosts exist and is considered to be the most universally distributed Lepidoptera. It is highly migratory and wind-borne adults can travel long distances to invade crops in other regions, countries and continents. Immature stages also hitchhike on plant parts and can establish in new areas. Several studies have examined the potential mechanisms underlying the development of insecticide resistance in *P. xylostella* (Sonoda, 2010) (Kim *et al.,* 2012) (Dukre *et al.,* 2009) one of the proposed mechanisms is metabolic resistance through the multifunctional glutathione S-transferases (GSTs, EC2.5.1.18) Glutathione conjugation is known to be a major pathway for the detoxifcation of xenobiotics as well as for the homeostasis of endogenous compounds.

Helicoverpa armigera is a polyphagous pest of agricultural crops. *Helicoverpa armigera* is a pest of major importance in most areas where it occurs, damaging a wide variety of food, fibre, oil-seed, fodder, commodity and horticultural crops. Its predilection for harvest-able parts of essential food and high-value crops like cotton, tomato, pulses and tobacco confers a high economic cost to its depredations. The high level of control required under these circumstances, and the absence, in most situations, of adequate natural control means that chemical, or at best integrated control methods usually need to be adopted.

The voracious caterpillars of *Helicoverpa armigera*an feed on leaves and stems buds, inflorescence, berries, pods, capsules etc, Young in-stars, however, may disappear completely inside, so they are sometimes not discovered before the produce (e.g. tomatoes) is processed (Harish, 2008).

Esterases, Glutathione S-transferases and Cytochrome P450-mediated monooxygenases are enzymes that facilitate the insects to metabolize different kind of toxins. Insecticide detoxification helps in understanding the mechanism of insecticide resistance, hence the development of a sound resistance management strategy. Detoxification can be divided into phase I (primary) and phase II (secondary) processes. Classification of Esterases is difficult because of their overlapping substrate specificity. According to Aldridge classification (Montella *et al.,* 2012), esterases inhibited by paraoxon in a progressive and temperature-dependent manner are called *B-esterases* and those which are not inhibited are *A-esterases*.

Molecular docking is a method which predicts the favored relative orientation of one molecule (key) when bound in an active site of another molecule (lock) to form a stable complex such that free energy of the overall system is minimized. In a simple definition, docking is a technique that is used to predict how a protein (enzyme) interacts with small molecules (ligands). It exploits the concept of molecular shape and physicochemical complementarity.

The ability of a protein (enzyme) and nucleic acid to interact with small molecules to form a supramolecular complex plays a major role in the dynamics of the protein, which may enhance or inhibit its biological function. The behavior of small molecules in the binding pockets of target proteins can be described by molecular docking. The method

aims to identify correct poses of ligands in the binding pocket of a protein and to predict the affinity between the ligand and the protein.

Based on the types of ligand, docking can be classified as

I. Protein–small molecule (ligand) docking
II. Protein–nucleic acid docking
III. Protein–protein docking

In this study, we focused on, the role of enzymes by enzymatic activity and its mechanism in detoxification of insecticide by in-vivo and in-vitro methods. GST and esterase are responsible for degradation of insecticides in diamondback moth, *Plutella xylostella* and *Helicoverpa armigera*. because these insect population showing the increased resistance to insecticides.

Materials and Methods

Chemicals used

All the chemicals used in the experiment were purchased from Sigma-Aldrich, India. Such as Bovine serum albumin, Reduced glutathione ,1 chloro 2,4 dinitrobenzene (CDNB), á-Naphthyl acetate, á-naphthol, Coomassie brilliant blue, Fast blue RR, Nitro Blue and Phenazine methosulphate.

Collection of insects

The field and reared population of *Plutella xylostella a*nd *Helicoverpa arimgera* third instar larvae collected.

The lab reared population of *Plutella xylostella* and *Helicoverpa arimgera* collected from mass production unit of NBAIR, ICAR –Hebbal, Bangalore. Which are reared on cauliflower and tomato leaves, collected during seasonal susceptible population from Doddaballapur, Bangalore rural district (Karnataka 35.2 N, 10.6 E)

The field larvae collected in cauliflower and tomato leaves during March 2019 from Doddaballapur, Bangalore rural district, (Karnataka 35.2 N, 10.6 E)

Sample preparation for enzyme assay

Enzyme activities of Esterase and GST was analyzed by using group of five, third instar larvae which were homogenised on ice in 1 ml of phosphate buffer (0.1 M, pH 6.5) containing 0.1% Triton X 100.The homogenate were then centrifuged at 15,000 g for 15 minutes at 4°C.The supernatant was used as an enzyme source for total protein quantification by enzymatic analysis and also Poly acrylamide gel electrophoresis (PAGE)

Protein estimation

The protein content in the enzyme extract was determined by employing Lowry's method using Bovine serum albumin (BSA) as standard. (Lowry *et al.,* 1951). The absorbance was taken at 660 nm, the slope was generated using standard graph and the amount of protein present in extract was calculated.

Esterase assay

Esterase activity was quantified according to the method of (Gomori, 1953) as modified by (Van Asperen, 1962). The absorbance of reaction mixture was measured at 600 nm using Shimadzu, UV 1700 spectrophotometer. The Enzyme activity was determined by using standard curve of α-naphthol.

Glutathione-S-Transferase assay (GST)

GSTs activity was assayed Spectrophotometrically at 25°C with reduced (GSH) and 1- chloro-2, 4-dinitrobenzene (CDNB) as substrates. The reaction mixture was prepared using 2.77ml of 0.1M phosphate buffer, pH 6.5, 50 of 50 mM CDNB in ethanol and 150 µl of 50 mM reduced glutathione with 30 µl of crude enzyme extract. The optical density at 340 nm was recorded at 0 to 5 minute interval in spectrophotometer (Shimadzu, 1600). The increase in absorbance was observed, the specific activity was calculated.

Polyacrylamide Gel electrophoresis (PAGE)

SDS-PAGE was carried out using Bio-Rad vertical slab system with 10% gel to enumerate esterase isozymes and GST enzymes from the homogenates of susceptible and resistant populations of *Plutella xylostella* and *Helicoverpa arimgera* insect larvae. Based on the protein concentration, about 20 µl of each insect (lab, field) population sample was loaded in to the wells. The gel was run at 100 V, once the samples moved ¾th of the gel, the gel was taken and stained in solution containing 0.4% α naphthyl acetate prepared in acetone and 0.1% fast blue B salt in phosphate buffer (40mM , pH 6.5) and incubated in dark for 5-10 minutes . After running, gel was placed in 0.1 M assay buffer containing 4.5 mM GSH, 1.0 mM CDNB, and 1.0 mM nitro blue tetrazolium, the gel kept at 37°C under gentle agitation. After 10 min, gel was washed twice with water and incubated at room temperature in 0.1 M Tris–HCl buffer, pH 9.6, containing 3.0 mM phenazine methosulfate. After 5 min, the gel surface was observed for the staining pattern. Then the gel was washed thoroughly with sterile water and kept in de-staining solution for overnight for de-staining and later observed for the respective bands of protein samples.

The Current study has been carried out to understand the mechanism of the Insecticide resistance in *Helicoverpa armigera* and *Plutella xylostella* so in order to emulate the insecticide treatment with Diclorovas, accessed and downloaded the same chemical compound from ZINC small molecule databasehttps://zinc.docking.org/ prepared this chemical compound1853865 (lead Molecule) further for docking using Autodock 4.0 as per the standard In-silico docking method as follows in the

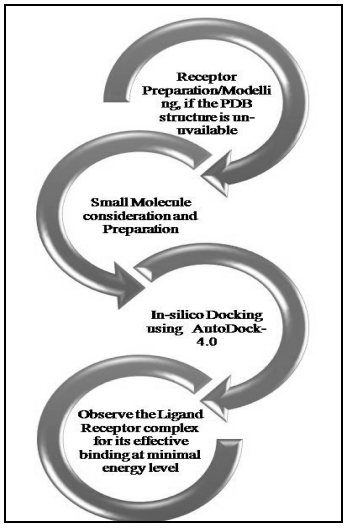

Fig. 1: Steps Showing the Protein Ligand Docking carried out in the current study

The insect metabolic enzyme Esterase and GST were obtained from NCBI database and were treated as receptors in the mechanism. Genbank ID AF065061.1 for Esterase in *H. armigera* and XP_011568923.1 for *P. xylostella* has been considered, further GST (2GST) protein has been considered and obtained from Genbank portal and used as a Homologous common receptor for both the considering insect pests.

Result and Discussion

Detoxifying enzyme activity

Standard graphs

Bovine serum albumin was used as a standard protein (Fig. 2) and with BSA a reference protein was estimated in all the populations of *Helicoverpa armigera* and *Plutella xylostella* used in the present study for determining insecticide resistance mechanism.

Similarly to determine the unknown concentration of Esterase and GST-Glutathione-s transferase enzyme activity, standard graphs were prepared using respective reference standard samples α naphthol (Fig. 3).

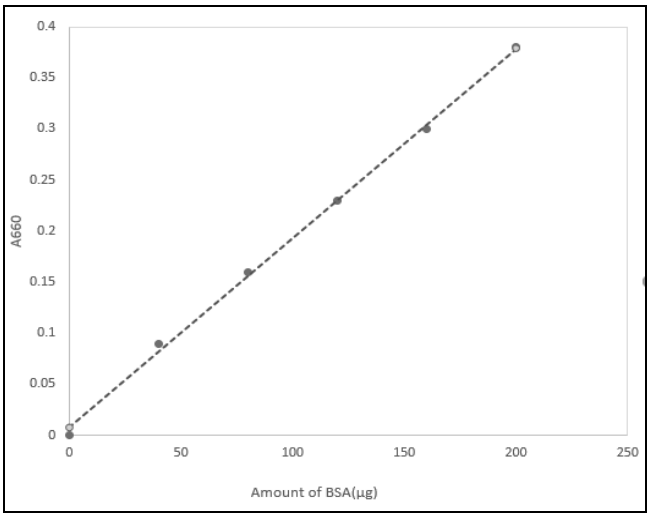

Fig. 2: Graph plotted for the estimation of protein using Bovine serum albumin as a standard protein

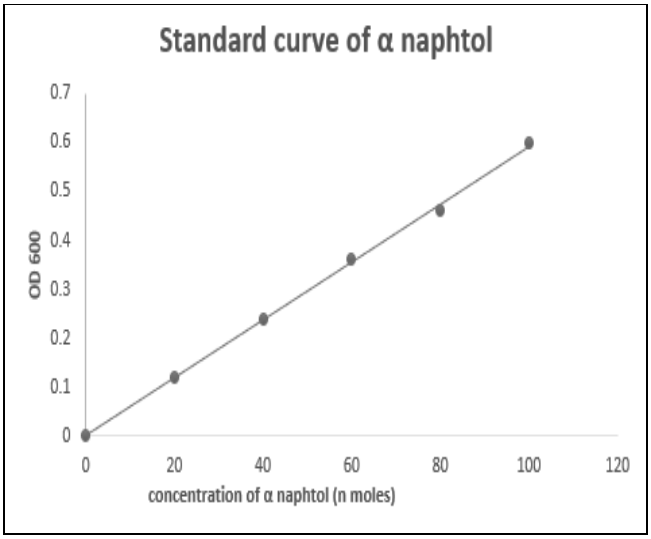

Fig. 3: Graph plotted to determine the activity of esterase and GST activity by taking known concentration of á naphthol

GST-Glutathione S-Transferse enzyme in *Helicoverpa armigera* and *Plutella xylostella*

Glutathione S-Transferse enzyme in *Helicoverpa armigera* and *Plutella xylostella* was determined, in the third instar insect larvae using CDNB as a substrate. In insects, GST's are classified into two groups, class I and class II respectively. The majority studies of insect GST's indicated the genetic and biochemical mechanisms in detoxifying the insecticide and developing resistance.

- GST activity was increased in the field populations of both *Helicoverpa armigera* and *Plutella xylostella* insect (Table 1). The increased activity was observed in the field population, when compared with susceptible population, because there is no glutathione conjugation occurred in susceptible population. Hence there is a 2 fold increase of enzyme in the resistant population. i.e. the activity of lab population of *Plutella xylostella* showed 500 µmoles/min/mg, and that of field population is 1430.63 µmoles/min/mg. Similarly the enzymatic activity of *Helicoverpa armigera* LAB reared showed 1818 µmoles/min/mg but field population showed 3054 µmoles/min/mg. This is because GSTs are responsible for many organophosphate degradation (Hayes J.D and Wolf C.R, 1988),the conjugation of glutathione to organophosphate insecticides results detoxification by different pathways. In some of the cases the glutathione reacts with leaving group, e.g. the degradation of parathion and methyl parathion in the diamondback moth *Plutella xylostella* (Chiang and Sun, 1993). In 1974, the first GST based resistance in mosquitoes was analysed, the author (Prapanthandara *et al.,* 1995, 1996, 2000a) demonstrated that GST's, when they present at highest amounts have an important role in degradation mechanism of insecticides.

Pattern of Glutathione S -Transferase using native PAGE

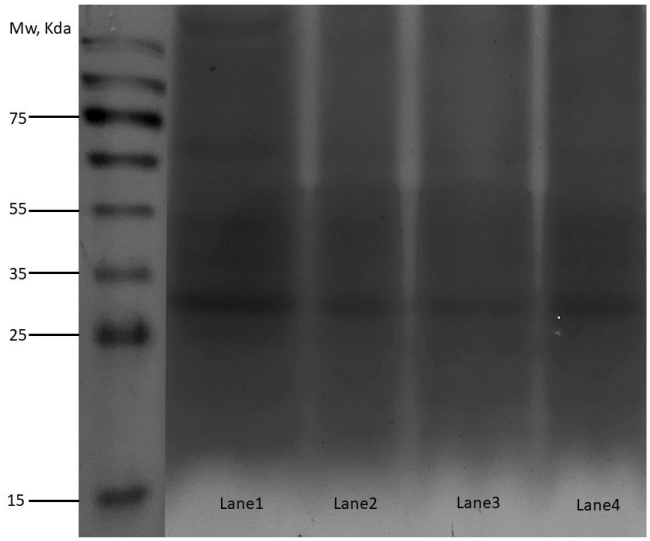

Fig. 4: SDS PAGE of GST enzyme of *Helicoverpa armigera* and *Plutella xylostella.*

Lane-1: LP of HA, Lane-2: FP of HA, Lane-3: LP of PX, Lane-4: FP of PX

GST activity visualized on native PAGE revealed GST bands. Thepresence of bands in the gel indicated that the substrate α-naphthyl acetate was being hydrolysed by GST, which indicated the presence of glutathione transferases in

insects; the band patterns of each population of two insects differed from the laboratory to field population (Fig. 3). Clear Bands were observed in the field population as well as lab population but the band intensity is greater in case of FP- Field Population of *Helicoverpa armigera* and *Plutella xylostella*, this indicates that insecticide resistance mechanism for degradation of organophosphate- Dichlorvos is easily detoxified by the field population because of highly expressed resistance mechanism. In the conjugation reaction, the active site residue of the GST interacts with GSH sulfhydryl group (-SH) to generate the catalytically active thiolate anion (GS-). This nucleophilic thiolate anion is then capable of attacking the electrophilic centre of any lipophilic compound to form the corresponding GS-conjugate (Armstrong, 1997). The conjugation neutralizes the electrophilic sites of the lipophilic component leading to its detoxification. The product obtained after conjugation is more water soluble and therefore readily excretable from the cells (Enayati *et al.,* 2005) via glutathione S-conjugate export pump and other mechanisms.

Esterase enzyme in *Helicoverpa armigera* and *Plutella xylostella*

Pest resistance to pesticide is also hereditary characteristic (Maa and Liao, 2000), because of the repeated use of pesticides, individuals those with great resistance increase their proportion gradually. Esterase enzyme activity in *Helicoverpa armigera* and *Plutella xylostella* is important for the degradation of xenobiotic compounds, and was determined in the third instar insect larvae. In this study the correlations between enzymes by its enzymatic activity were analysed. The results showed there is an increase in GST enzyme in field population compared to lab reared/ susceptible population, but in case of esterase enzyme, the increase of activity was observed in lab population when compared to field population (Table 1). i.e. the activity of lab population of *Plutella xylostella* showed 0.0036 µmoles/ min/ml, and that of field population is 0.0029 µmoles/ min/ml. Similarly the enzymatic activity of *Helicoverpa armigera* LAB reared showed 0.0084 µmoles/ min/ml but field population showed 0.0055µmoles/min/ml. This is because the metabolic capacity is strongly related to the activities of enzymes carboxyl esterase's and GST's (Hung and Sun, 1989) (Kao and Sun, 1991). Some reports indicated that an increase in temperature would inhibit the activities of enzymes, hence the variations of enzymatic activity of Esterase enzyme was observed in increase in laboratory reared and decrease in field population.

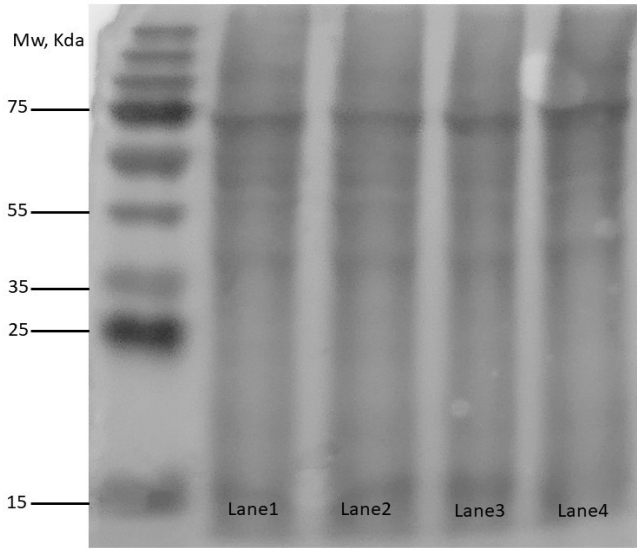

Fig. 5: SDS PAGE of Esterase enzyme of *Helicoverpa armigera* and *Plutella xylostella.*

Lane-1: FP of HA, Lane-2: FP of PX, Lane-3: LP of HA, Lane-4:LP of PX

Estearse activity visualized on native PAGE revealed Estearse bands. In insects, the potent inhibitors of AChE are organophosphates. These compounds inhibit the activity of AChE by forming a stable covalent intermediate, preventing the enzyme to hydrolyze acetylcholine. So the Decrease in Esterase activity in field population clearly says that these esterases are used to hydrolyse the ester bonds of insecticide.

The presence of bands in the gel indicated that the expression of protein in the sample varying because of degradation mechanism of insecticides. The band pattern of each population differs from the laboratory/susceptible population (Fig. 4) to field population. Clear Bands were observed in the both population, but the intensity of protein is greater in case of lab Population of *Helicoverpa armigera* and *Plutella xylostella*, this indicates that expression of protein which is responsible for detoxification is high in lab population because of repeated exposure of insect to Dichlorvos insecticide.

Table 1: Protein concentration and enzyme activity observed in crude extract of *Plutella xylostella* and *Helicoverpa armigera.*

Name of an Insect	Insect Population	Esterase activity (µmoles/min/ml)	GST activity(µmoles/min/mg protein)
Plutella xylostella	LAB	3.16 x 10-4	507
	FIELD	2.9 x 10-4	1,430.63
Helicoverpa armigera	LAB	84 x 10-4	1,815.40
	FIELD	55 X 10-4	3,054.68

During the In-silico docking it has been observed that in Esterase in *Helicoverpa armigera.* The binding energy of this dock is -2.21. The active pocket includes amino acids Gly 122, Ser 199, ILE 392, SER 224, GLY 225, ILE 233, SER 226. THR 230, ALA 391. GLY 121, HIS 434 and TYR 200

The binding energy of this dock is -2.21 which is contributed by Vanderwall's forces of attraction were contributed GLY 122, SER 199, ILE 392, GLY 225, ILE 233, SER 226. THR 230, ALA 391 AND GLY 121. TYR 200 forms pi sigma bond with two chlorine atoms of dichlorvos.

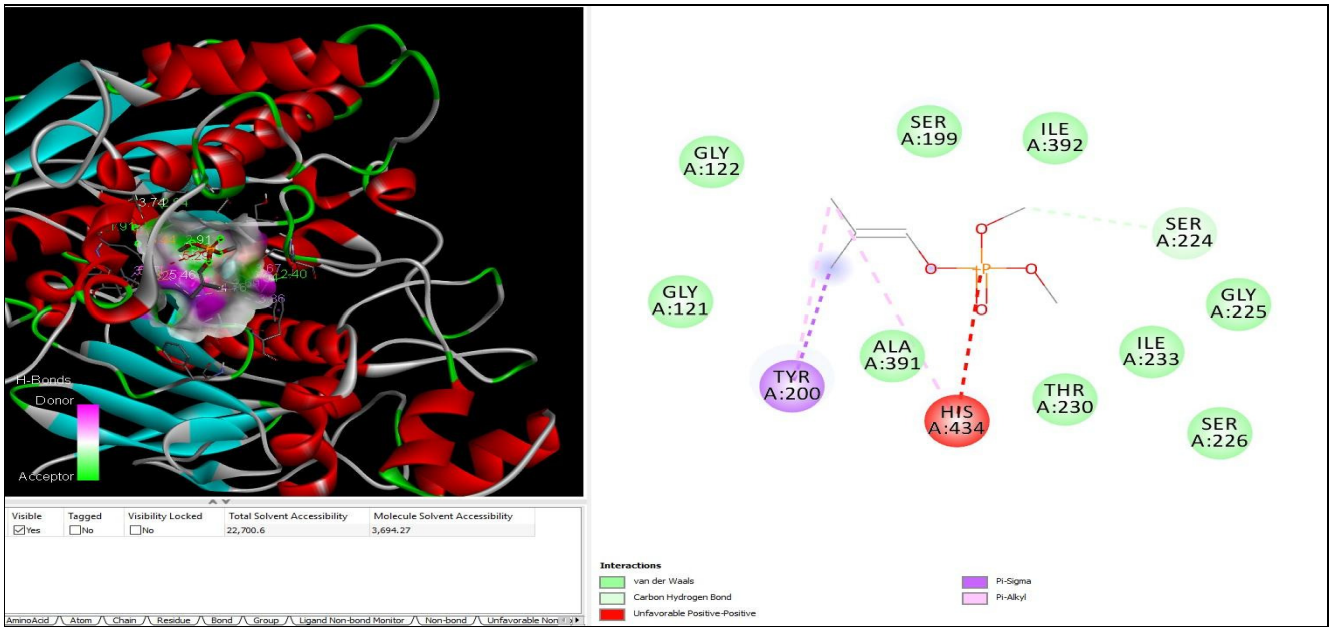

Fig. 6 : Showing the docked complex of esterase in *H.armigera* with dichlorvos

Table 2: Showing Binding energy of the docked complex between esterase of *H.armigera* and Dichlorvos

Rank	Sub-Rank	Run	Binding Energy	Cluster RMSD	Reference RMSD
1	1	2	-2.21	0.00	26.93
1	2	1	-2.18	0.77	27.00
1	3	6	-2.15	1.40	26.66
1	4	4	-1.49	0.82	26.64
1	5	9	-0.25	1.42	26.80
1	6	8	+0.91	1.82	26.09
2	1	3	-1.94	0.00	25.97
3	1	7	-1.08	0.00	33.13
4	1	10	+4.10	0.00	33.54
5	1	5	+30.55	0.00	33.26

In case of esterase *Plutella xylostella* The binding energy of this dock is -4.83the bonding has been established at the amino acids present at the active pocketLEU 139, VAL 140, LEU 436, GLY 210, HIS 126, GLY127, ILE 125, GLY 206, TYR 124, ASP 134, TYR 440, ALA 205, GLU 232, The binding energy of this dock is -4.83 which is contributed by Vanderwalls forces of attraction were contributed by ASP 134, TYR 440, ALA 205,GLU 232, TYR 124, GLY 206, ILE 125,GLY 210 AND HIS 126. GLY 127 forms hydrogen bond with oxygen of phosphate moiety of dichlorvos which is going to contribute larger inhibition of dichlorvos to esterase enzyme, the amino acid LEU 436, Val 140 and Leu 139 forms alkyl bond with two chlorine atoms of dichlorvos

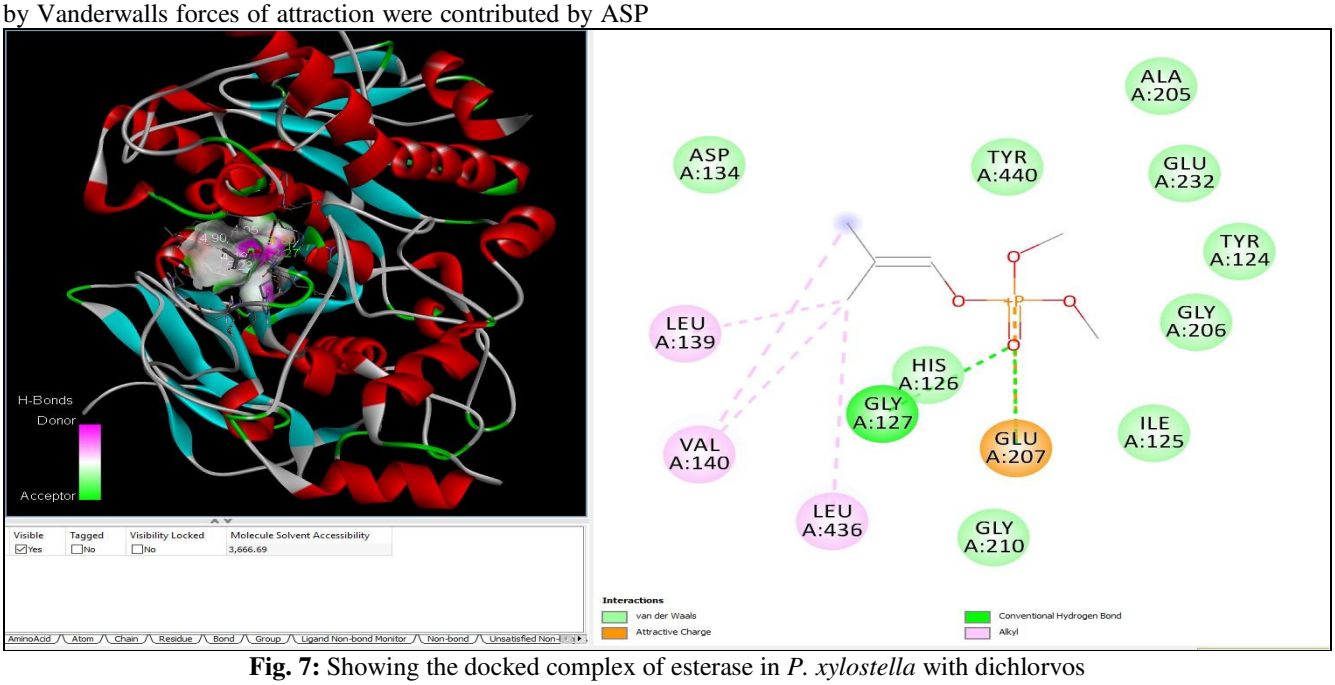

Fig. 7: Showing the docked complex of esterase in *P. xylostella* with dichlorvos

Table 3: Showing Binding energy of the docked complex between esterase of *P. xylostella* and Dichlorvos.

Rank	Sub-Rank	Run	Binding Energy	Cluster RMSD	Reference RMSD
1	1	8	-4.83	0.00	27.52
1	2	5	-4.67	0.70	27.63
1	3	10	-4.57	0.90	27.44
1	4	9	-4.47	1.26	27.12
1	5	1	-4.18	1.62	27.95
1	6	7	-4.05	1.63	27.94
1	7	4	-3.94	1.07	27.26
1	8	2	-3.93	1.64	27.67
1	9	3	-3.31	1.73	27.76
2	1	6	-4.39	0.00	27.39

The homologous GST showed the binding energy of this dock is -4.27. the active site pocket includes the amino acids TYR 115, SER 209, TYR 6, LEU 12, PHE 208, GLY 11, ILE 207, ILE 111, A-MET 34, VAL 9, and TRP 7. The amino acids involved in carbon hydrogen bonding are LEU 12, PHE 208, GLY 11, ILE 207, ILE 111, A-MET 34, VAL 9. The amino acids involved in Vander Waal bonding are TYR 115, SER 209, TYR 6 with oxygen atoms of phosphate group of dichlorvos.

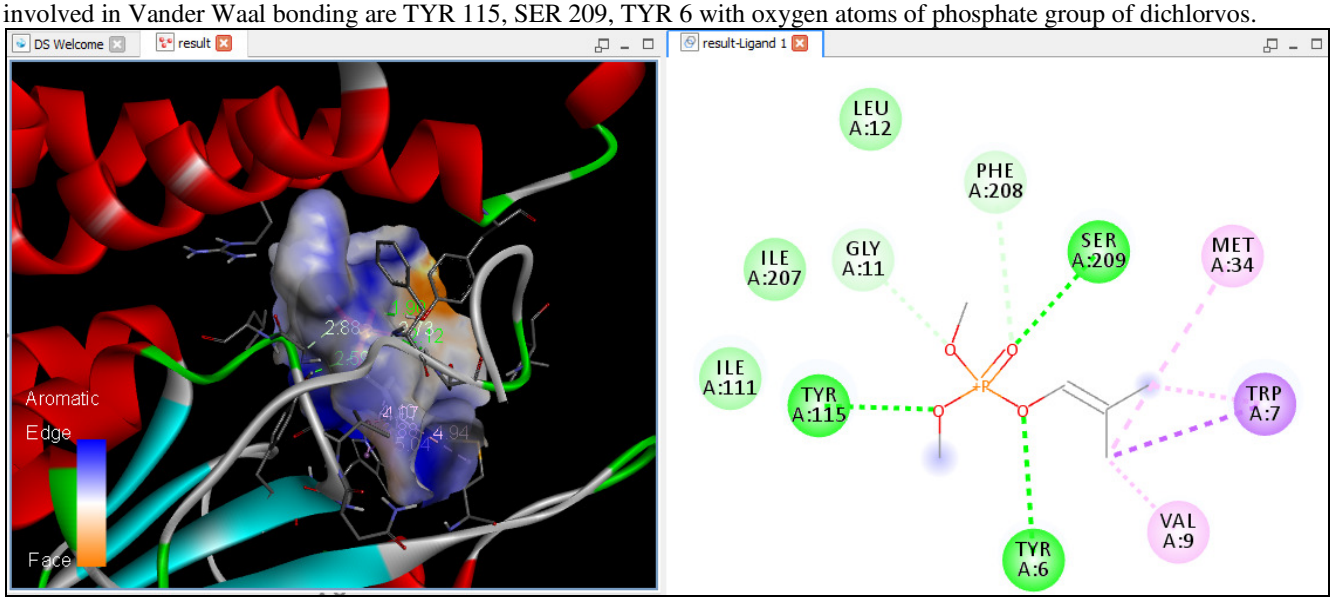

Fig. 8: Showing the docked complex of GST with dichlorvos

Table 4: Showing Binding energy of the docked complex between esterase of GST with dichlorvos.

Rank	Sub-Rank	Run	Binding Energy	Cluster RMSD	Reference RMSD
1	1	4	-4.27	0.00	30.75
1	2	5	-4.20	1.63	30.75
2	1	9	-4.12	0.00	30.46
3	1	7	-4.06	0.00	41.27
3	2	3	-3.89	1.06	41.00
3	3	10	-3.82	1.30	40.97
3	4	1	-3.65	1.84	40.52
4	1	2	-4.04	0.00	40.56
5	1	6	-3.83	0.00	29.75
6	1	8	-3.72	0.00	40.83

In the view of the In-silico docking session, Esterase and Glutothione S transferase, it clearly indicated that esterase activity in *P. xylostella* has reflected in binding energy -4.83, on the other hand *H. armigera* is showing the value of -2.21. Lesser the binding energy expressed in the *P. xylostella* conveys that higher rate of interaction in-turn performing the degradation activity against Dichlorvos and exhibiting the higher resistance, where as in *H. armigera* showing the higher binding energy in-turn the less efficiency in the degradation of Dichlorvos so *H. armigera* is showing less resistance towards this insecticide.

Here we considered GST is homologous protein which has shown the binding energy of -4.27 which is showing restrained affinity in binding insecticide Dichlorvos which in-turn not exhibiting enzyme mediated Insecticide resistance mechanism.

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