

Pesticide Application Inhibit the Microbial Carbonic Anhydrase Mediated Carbon Sequestration in a Soil Microcosm

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Abstract

Heterotrophic system for carbon sequestration is gaining importance in the recent decades. Carbonic anhydrase (CA) is a major enzyme involved in carbon sequestration and biomineralization process. In this paper, we evaluate the effect of pesticide on CA activity using inhibitory assay. 2,4-D, being one of the most extensively used pesticide, being deleterious to soil health, its usage should be minimized to regain the soil health. Maximum inhibitory constant (K_i) was observed for 5% 2,4-D (49.53 mM) followed by 5% glyphosate (43.92 mM). The maximum K_m increase with increase in pesticide concentration by 3.05-fold was in case of glyphosate which was higher than that of 2,4-D (2.08-fold) and dichlorvos (2.38-fold). Moreover, we evaluated the carbon sequestration using CA enzyme in the soil microcosm. In the present study, we identified the negative impact of 2,4-D on carbonic anhydrase produced by *Bacillus halodurans* PO15. The inhibition was a mixed type and had significantly lowered the carbon reduction to about $2.38 \pm 0.17\%$ in a soil microcosm study. Based on the molecular docking, the inhibition was contributed due to weak H-bonding interaction with amino acid residues (Gly65, Gly95, Val147, Ser150 and Gly65, Ser146 and Ser150).

Keywords: Pesticides; molecular docking; carbonic anhydrase; sequestration .

Introduction

Anthropogenic activities have significantly contributed to increased atmospheric greenhouse gas (GHG) concentrations over last centuries. There are many natural processes contributing to the storage and cycling of carbon. However, the stability and sequestration of these large carbon sinks, in oceanic and terrestrial environments are certainly influenced by the inhabitant microbial community. Managed carbon pools like forest, agriculture lands also are involved in the carbon storage in global ecosystems and it is essential to understand how these they influence atmospheric CO₂ concentrations in reducing uncertainty in climate models and in the long-term sequestration capacity of those pools (Carbon Sequestration 1999). The Department of energy, US mission of global carbon management reports the importance of a comprehensive understanding of terrestrial and marine microbial communities linking to their role in carbon sequestration. This might help in enhancing their capabilities to develop microbe-based strategies for capturing and sequestering atmospheric CO₂ and to assess the potential effectiveness and adverse ecological impacts of proposed carbon-sequestration technologies. Moreover, these microbial systems act as sensitive indicators of environmental change which help in monitoring process.

Soil is the largest carbon pool in the terrestrial biosphere containing twice as much carbon as in the atmosphere, with half the concentration accumulated within the topsoil (0–30 cm) (Amundson 2001; Gleixner 2013; Lal 2019). In the terrestrial ecosystem, agricultural lands constitute about 40% of the area. Soil microorganisms act as gatekeepers for soil–atmosphere carbon exchange by balancing the accumulation and release of soil organic matter (SOC) (Malik et al. 2018). These microorganisms regulate the soil carbon fluxes by release of organic carbon through decomposition of organic matter (Schimel and Schaeffer 2012; Gleixner 2013). Hence, a better understanding of microbial regulation may be critical in projecting changes in SOC, thereby improving predictions of climate change feedbacks (Wiederet al. 2013; Bradford et al. 2016).

The unscientific application of agrochemicals like pesticides and chemical fertilizers actually hamper the normal microbial community of the land. Further, it also disturbs the metabolite production by microbes and ultimately, the biogeochemistry of the soil. Increasing soil carbon is a cost effective means to sequester carbon that provides a range of benefits which include enhanced water holding capacity, water infiltration rates, along with reduction in bulk density, erosion potential, and salinization. This also reduce the demand for agro-chemical applications (Favoino and Hogg 2008).

Soil-dwelling microorganisms are affected not only by active substances but also by their degradation products, which could be more toxic than the parent compounds (Tixieret al. 2002). Some pesticides are slowly degraded in soil, which can lead to their accumulation in concentrations that are harmful for soil-dwelling microorganisms (Muñoz-Leozet al. 2013). Numerous research studies have demonstrated that continuous use of pesticides can induce changes in soil ecosystems (Araújo et al. 2003; Cycoń et al. 2013). Pesticides can affect soil microbes, which may change their abundance and diversity (Ratcliff et al. 2006; Cycoń and Piotrowska-Seget 2009; Cycoń et al. 2010; Baćmaga et al. 2014). This may also change the microbial and enzymatic activity of soil (Kucharski and Wyszowska 2008; Zhang et al. 2010; Baćmaga et al. 2012). Although many reports are available on the carbon sequestration process using heterotrophic bacterial enzymes, the impact of pesticides on these enzymes and on their process is largely unexplored. In this paper, we evaluate the inhibition of carbonic anhydrase (CA) in carbon sequestration in a soil microcosm by the pesticide application.

Materials and Methods

Chemicals

p-Nitro phenol acetate (Sigma Aldrich, USA), pesticides namely 2,4-D, Dichlorvos and Glyphosate were some available insecticides and herbicides in the local market. Due to the banning of other chemicals, we used only the presently available chemicals for the experiments.

Bacterial Enzyme

Bacillus halodurans PO15, a potential bacterial isolate with high carbonic anhydrase (CA) activity identified in our previous work for which enzyme characterization and structural prediction was carried out using *in silico* methods. We used the purified fraction of the CA for the experiments.

Inhibitory study of CA from *Bacillus halodurans* PO15

Carbonic anhydrase esterase activity was determined by analysing the p-nitro phenylacetate (p-NPA) hydrolysis as per the standard protocol (Victoria et al. 2009). Briefly, activity was determined in a reaction mixture containing 0.5 mL of freshly prepared 3 mM p-NPA in aqueous 3% (v/v) acetone and 0.85 mL of water at 25°C. The uncatalyzed rate of the reaction was determined by adding 0.15 mL of 100 mM potassium phosphate (pH 7.0) containing 1 mM zinc sulfate and the change was recorded at A_{348} per min (ϵ 5000 M⁻¹cm⁻¹). After 2 min, 15 µl of enzyme solution was added, and the catalyzed reaction was monitored for additional 3 min. The enzyme substrates were prepared at different concentrations (1-5 mM) and subjected to enzyme assay. The enzyme- substrate

interaction was illustrated using the Line-weaver Burk (LB) plot. K_m and V_{max} values were calculated from the LB plot. K_i values were calculated based on the equation 1:

$$K_i = \frac{[E][I]}{[EI]} \quad (\text{equation 1})$$

Based on the K_m and V_{max} value changes, the type of inhibition exhibited by the pesticides were also predicted.

Xenosite analysis of xenobiotic toxicity

Though the effect of insecticides and herbicides accumulation in soil has been studied, in the present study, *in silico* models were used for the toxicity analysis. XenoSite analysis provides prediction for small molecule biochemistry especially about the human *in vivo* metabolism and reactivity of small molecules. In the server tools, Xenosite Reactivity 2.0 tool enabled the prediction of DNA adducts formation for each metabolite (Hughes et al. 2015; 2016).

Molecular Docking of CA with Pesticides

Ligand retrieval

The structures of the selected insecticides (Dichlorvos) and herbicides (2,4-D, and Glyphosate) were retrieved from the PubChem database for docking calculation. The selected 3D structure of the ligands was retrieved from PubChem compound database in SDF format followed by conversion in the PDB format and optimization using Discovery Studio. Prior to docking analysis, active sites were predicted using Computed Atlas of Surface Topography of Proteins (CASTp) server (Tian et al. 2018).

Molecular Docking Study for CA inhibition by Pesticides

The modelled bacterial carbonic anhydrase structure (*Bacillus halodurans* PO15) was used as target enzyme and tested with 3 ligands. Docking analysis of the selected target was conducted by the computational stimulation of target with ligand using the docking software Discovery studio. The protein structure was prepared by adding missing hydrogen and the binding site of the protein were predicted from receptor cavities. The ligands were prepared and subjected to docking by library docking and centered docking as C-docking.

Effect of CA in Soil Microcosm

A soil microcosm was designed in accordance with Salam et al. (2015) with slight modifications for testing the efficacy of potential bacteria and its carbonic anhydrase on CO_2 mitigation. Soil samples were collected from Mangalavanam mangrove ecosystem ($9^\circ 59' 13.4''$ N

and 76° 16' 26.1" E) using a PVC corer at a depth of 10-12 cm, after removal of debris from the soil surface. For the microcosm, 20 g soil of fresh soil sample was added in each 125 mL reagent bottle and sterilized 3 times to remove any contaminants and to remove normal microbial flora. pH of sediment pore was observed (pH 6.5) and maintained a uniform pH for all microcosm setups. The sterility of soil sample was confirmed by adding a portion of the sterile sub-sample into nutrient broth media and checked for no visible bacterial growth after 24 hrs of incubation.

Twenty-two different experimental conditions were used and the details of each microcosm setup was enlisted in table 1. Sterile soil (SS) and unsterile soil (US) without addition of CAenzyme was made with and without pesticide fortification. The experimental setup without pesticide was used as control. To enumerate the effect of pesticide addition on bacterial abundance, the subsamples were taken from unsterile microcosm after pesticide addition and plates on to nutrient agar plates after serial dilution. In all the microcosms, the purified carbonic anhydrase of *Bacillus halodurans* isolate PO15 (50 U/mL) was added (10% v/w). Soil moisture was made uniform using sterile distilled water in case of controls. Unsterile soil microcosm was used to detect the soil respiration from normal microbial flora, whereas in sterile soil microcosm had no organisms for respiration. Approximately, 5% CO₂ gas was purged into the bottles and were sealed airtight. The microcosms were incubated in a water bath at 32°C for 24 hrs (ambient temperature recorded at the mangrove ecosystem). The concentration of CO₂ in each microcosm was read using a headspace CO₂ analyser (UniphosEnvirotronic Pvt. Ltd, India) and was expressed as % relative reduction. All experiments were carried out in duplicate unless otherwise mentioned and the values were expressed as mean ± S.D.

Results and Discussion

Effect of pesticides on microbial abundance

The total plate count was evaluated after the sediment samples were exposed to pesticides. The pesticides were added to soil samples at different dosage. From the study, it was found that dichlorovos reduced the plate count from 3.2×10^6 to 1×10^6 cfu/g. While glyphosate treatment reduced the counts to 1.2×10^6 cfu/g. The 2,4-D treated sample had only a low reduction (2.8×10^6 cfu/g) which might be due to a resistance developed in microorganisms due to extensive regional usage of 2,4-D. Pesticides can cause direct toxic or beneficial effects on microbial communities. Some microbes particularly bacteria may utilize pesticides as a source of nutrients facilitating their growth and survival, while some sensitive species may have some negative effects by pesticides (DeLorenzo et al. 2001; Russell et al. 2011). Toxicity of the pesticides may be influenced

by processes like leaching, run-off, degradation, adsorption, volatilization, plant uptake etc. (Pose-Juan et al. 2017). These processes also influence the amount of pesticide in the soil that is bioavailable for affecting microorganisms (Jacobsen and Hjelmsø 2014), which may be altered by the application of organic residues (Herrero-Hernández et al. 2011).

The influence of microbial diversity on soil functions is largely unclear (Wertz et al. 2006). Pesticide application has an impact on the microbial diversity of the soil. In previous studies, the growth-promoting bacteria in rhizosphere was reduced during glyphosate application (Zobiolo et al. 2011) and the raised the number of Gram negative bacteria following naproamide treatment (Cycoń et al. 2013). Effects have also been seen for insecticides (Zhanget al. 2009) and fungicides (Yang et al. 2012).

Inhibition Kinetics of Herbicide on CA of *Bacillus halodurans* isolate PO15

Inhibitory kinetics of CA from *B. halodurans* PO15 was studied using LB plot. Three different pesticides were used in different concentrations (1, 3, 5%) for the experiment. A higher pesticide concentration (above 5%) totally inhibited the enzyme activity. The K_m values were found to increase with increasing concentration of pesticides. Whereas, V_{max} values were found to decrease when pesticide concentration was increased. K_i values were calculated and represented in Table 2. K_i values showed an increasing trend with increasing pesticide concentrations (fig 1). The maximum K_m value was observed at 5% 2,4-D (44.97 mM) while the least was for 1% glyphosate (13.08 mM). The V_{max} values were least in case of 5% 2,4-D (91.74 $\mu\text{mol}/\text{mg protein}/\text{min}$) and the highest shown at 1% dichlorvos (232.55 $\mu\text{mol}/\text{mg protein}/\text{min}$). Maximum inhibitory constant (K_i) was observed for 5% 2,4-D (49.53 mM) followed by 5% glyphosate (43.92 mM). The maximum K_m increase with increase in pesticide concentration by 3.05-fold was in case of glyphosate which was higher than that of 2,4-D (2.08-fold) and dichlorvos (2.38-fold). Moreover, V_{max} value was decreased by 2.42-fold and K_i increased by 6.89-fold in case of 2,4-D. A low K_m value indicates that the enzyme requires a small amount of p-NPA to become saturated.

Hence, during minimal pesticide concentration, a maximum velocity is reached at a low concentration of p- nitrophenyl acetate. It is well known that enzymes catalyze almost all chemical reactions in the metabolism of the living systems, and many chemical substances including pesticides, fungicides, drugs and metal ions influence metabolism at low concentrations by decreasing or increasing enzyme activities (Ceyhünet al. 2011). The glyphosate, isopropylamine and dichlorvos treatments inhibited CA-II esterase activity in sheep stomach in a non-competitive manner (Kılınç et al. 2015). This was in agreement with our present observation where concentrations

of 1 and 3%, all the three pesticides exhibited non-competitive inhibition while at highest concentration tested, it showed mixed inhibition. However, some pesticides like Nuarimol™, Fenarimol™, and 2,4-D™ exhibited 50% inhibition of the enzymatic activity of erythrocyte carbonic anhydrase (CA) even at lowest concentrations (Sinanet al. 2007).

Though the effect of insecticides and herbicides accumulation in soil has been studied, in the present study, the same was addressed using *in silico* models. XenoSite analysis provides prediction for small molecule biochemistry especially about the human *in vivo* metabolism and reactivity of small molecules. In the server tools, Xenosite Reactivity 2.0 tool enabled the prediction of DNA adducts formation for each metabolite (fig 2). From the prediction, the dichlorvos had major deleterious effect on DNA, GSH and proteins than other two tested pesticides.

Molecular inhibition of CA

The residues placed in the active sites of the CA enzyme were involved in the inhibition process by formation of weak hydrogen bonds. The active site map of the CA is depicted in Fig 3. From the docking analysis 2,4-D showed highest docking score when compared to other ligands with Z-dock score of 88 and showed weak H-bonding interaction with Gly65, Gly95, Val147, Ser150 and Gly65, Ser146 and Ser150 respectively (Fig 4). The ligand Glyphosate showed higher binding affinity with target showed 3 strong H bonds and 6 weak hydrogen bonds (Table 3). The ligand glyphosate showed the strongest interaction with the target protein. The amino acids such as Gly 65, Ser146, and Ser150 may have a definite role in enzymatic activity.

Effect of agro-chemical on CO₂ sequestration in soil microcosm

In the soil microcosm experiment to demonstrate the effect of pesticides on CO₂ sequestration, a maximum CO₂ reduction ($84 \pm 0.54\%$) was observed in sterile soil + enzyme (SS+E) microcosm. In the unsterile soil with CA enzyme, the CO₂ reduction was only about $34.15 \pm 0.24\%$. This may be due to contribution of CO₂ emission from the microbial flora in the soil. In case of microcosms with addition of pesticides, there was decline in CO₂ reduction with increase in pesticide concentration. A maximum CO₂ reduction of $38.28 \pm 0.17\%$ was observed in unsterile soil with 1% glyphosate (US+E+Gly I) (fig 5). However, in case of 2,4-D and dichlorvos, the reduction of CO₂ reduction was significant with increasing pesticide concentration. A maximum inhibition of CO₂ reduction was observed in sterile soil with enzyme and 5% 2,4-D (SS+E+2,4-D III).

Primarily, enzyme production is an expensive in terms of energy consumption and nutritional requirement (Schimel and Weintraub 2003). Therefore, there may be a decrease in microbial enzyme production by microbes during the process of acclimatization to stress that may be by switching over

to new available resources for their survival (Schimel et al. 2007). In our study, we observed a reduced enzyme activity during pesticide application in soil from the *in vitro* inhibition assay and molecular docking results.

Pesticide application being an abiotic stress may lead to loss of function of the active site or change of enzyme conformation (Wallenstein et al. 2010). This activity reduction was observed in enzymes like cellobiosidase, chitinase, and phosphatase during abiotic stress (Wallenstein et al. 2010). The reduction of hydrolytic enzyme per biomass unit were reduced indicating a down-regulation of microbial enzyme production per unit of microbial biomass. In case of microcosms with addition of pesticides, there was decline in CO₂ reduction with increase in pesticide concentration. The CO₂ reduction rate was reduced to about $2.38 \pm 0.17\%$ in unsterile soil with 3% 2,4-D (US+E+2,4-D III). While in case of glyphosate and dichlorvos, the inhibition was low compared to 2,4-D.

The microcosm with maximum glyphosate exhibited about $11.90 \pm 0.32\%$ CO₂ reduction while for dichlorvos, it was $9.52 \pm 0.17\%$. Pesticides can affect soil microbes, leading to changes in their abundance (Baćmaga et al. 2014) and biological diversity (Ratcliff et al. 2006; Cycoń and Piotrowska-Seget 2009; Cycoń et al. 2010) as well as changes in the microbial (Zhang et al. 2010) and enzymatic activity of soil (Kucharski and Wyszowska 2008; Baćmaga et al. 2012). Glyphosate application have indirect impact on soil microbial functions resulting in a decrease in labile C input into the rhizosphere. This therefore decreases soil microbial activity leading to lower soil respiration and C cycling. Hence, glyphosate effects soil microbes in terms of heterotrophic respiration, the activity of soil C-cycling enzymes, and organic matter degradation and mineralisation (Rose et al. 2016).

Conclusion

In the present study, we identified the negative impact of 2,4-D on carbonic anhydrase, a carbon sequestering enzyme produced by *Bacillus halodurans* PO15. The pesticide application had a negative impact on the bacterial abundance and carbonic anhydrase activity. The inhibition was a mixed type at highest pesticide concentration tested and had significantly lowered the carbon reduction to about $2.38 \pm 0.17\%$ in a soil microcosm study. This forms the first report on demonstration of pesticide mediated inhibition of carbonic anhydrase, an important enzyme in carbon sequestration. Based on the molecular docking, the inhibition was due to weak H-bonding interaction with Gly65, Gly95, Val147, Ser150 and Gly65, Ser146 and Ser150 respectively residues.

Hence, it was concluded that pesticide application should be minimised for the environmental safety as its uncontrolled usage may also effect the carbon sequestration process in the soil.

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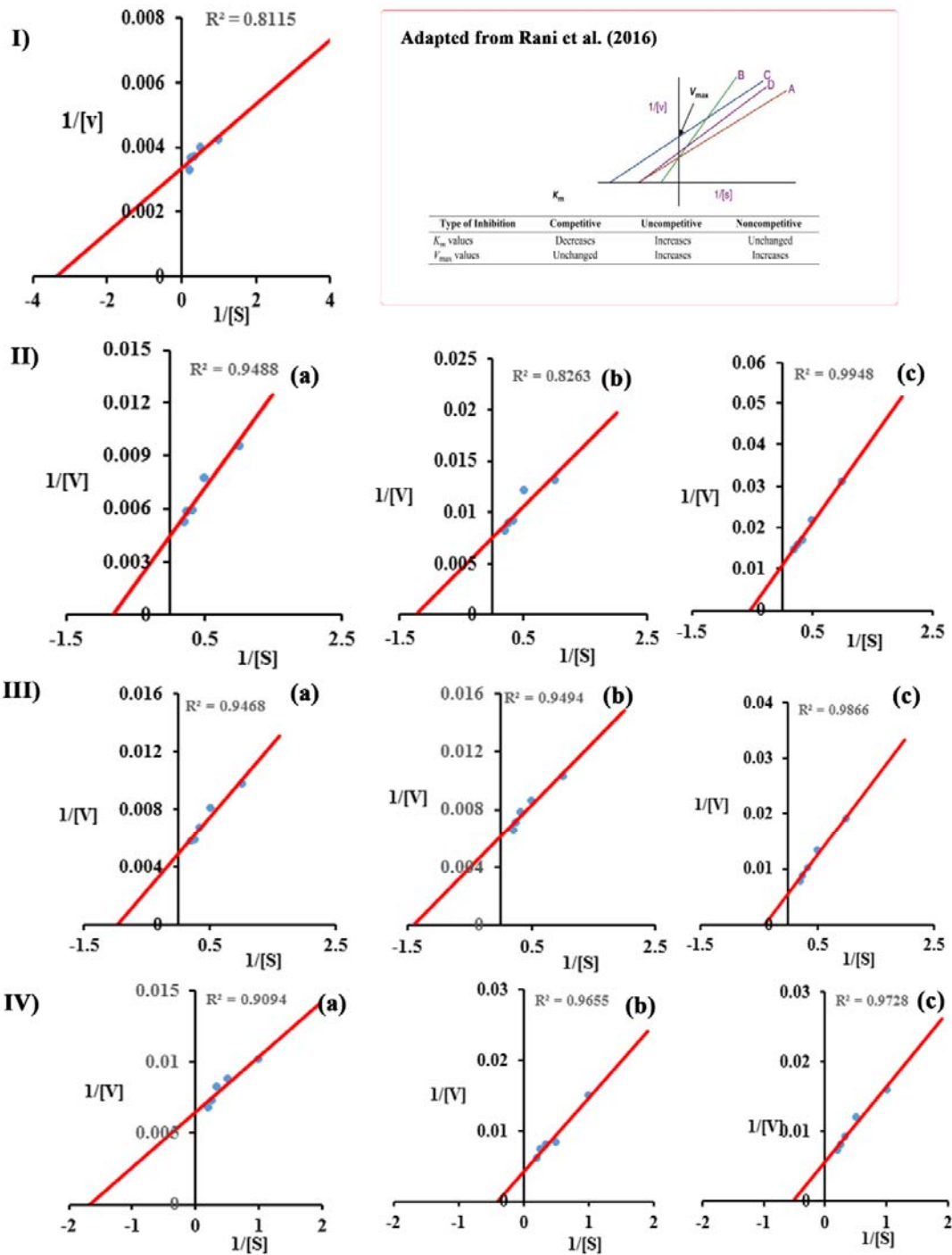


Fig 1. Determination of K_i value of agro-chemicals for *Bacillus halodurans* PO15 carbonic anhydrase (CA) enzyme by Line weaver-Burk plots. I) LB-plot of CA enzyme in absence of any inhibitors (insert picture –representing type of enzyme inhibition) II) 2,4-D III) dichlorovos and IV) glyphosate (a, b and c represent 1, 3 and 5% concentration respectively) ($1/[V]$ expressed in $\mu\text{mol/p-NPA/protein/min}$ and $1/[S]$ in mM)

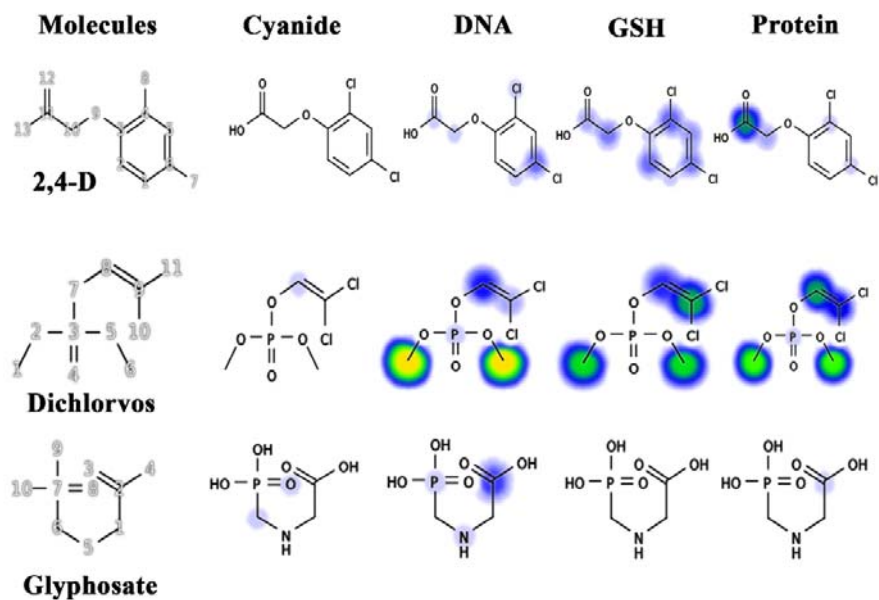


Fig 2. Xenosite prediction of toxicity of the three selected pesticides. The metabolic affected regions were shaded with a contour scale colour.

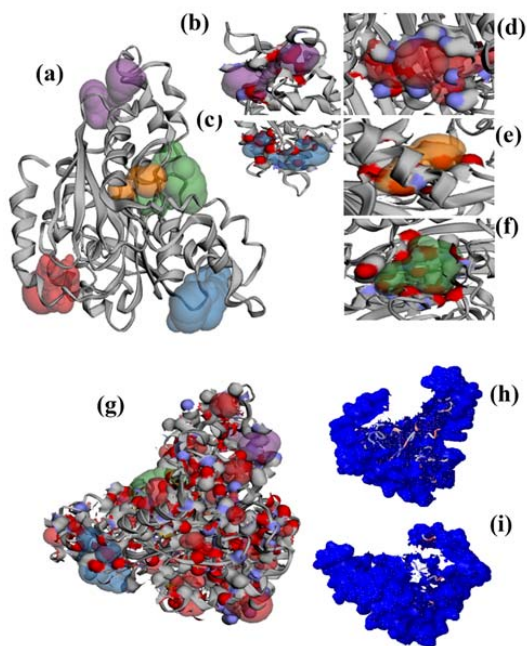


Fig 3. Carbonic anhydrase of *Bacillus halodurans* PO15 with its tertiary structure.

a) Representation of five major active sites of the enzyme (Coloured bubbles).

b-f) enlarged view of selected active sites. g) 72 active predicted using CASTp analysis and h & i) molecular surface structure of carbonic anhydrase

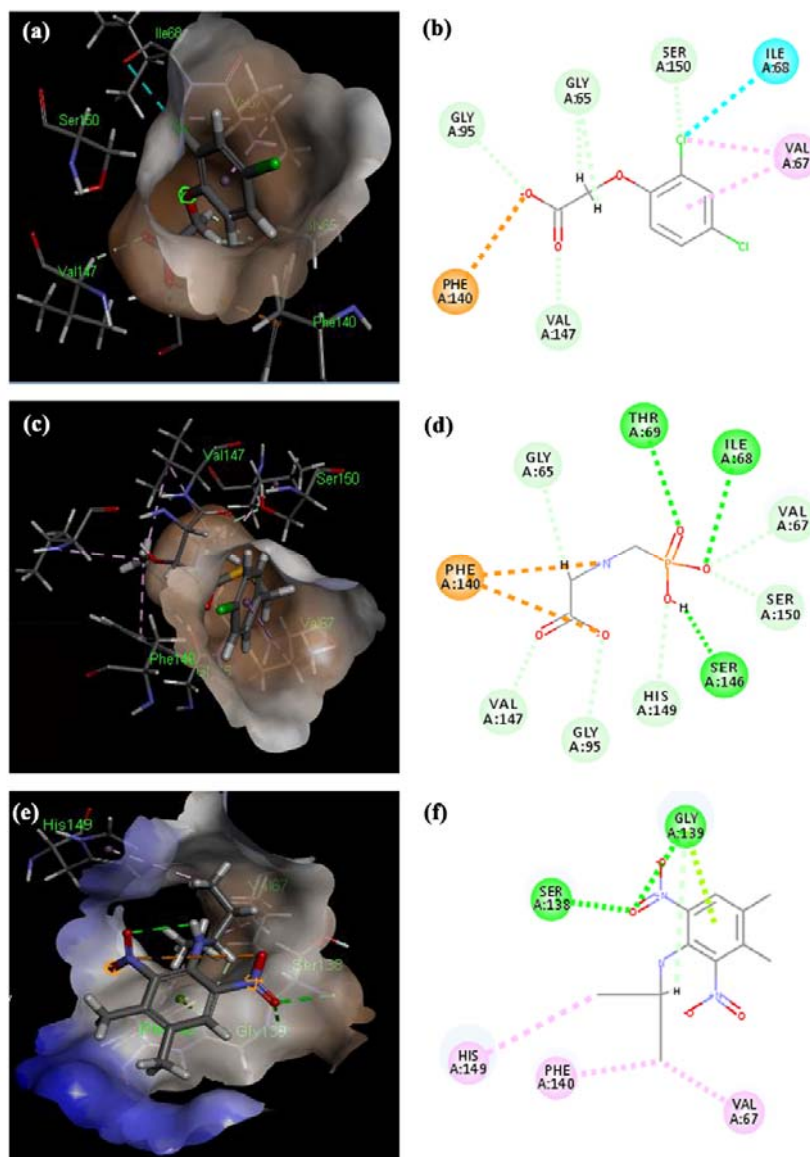


Fig 4. Binding mode of pesticides with *Bacillus halodurans* PO15 CA predicted by molecular docking and energy minimization. Molecular docking and 2D interaction diagram - (a) & (b) 2,4-D; (c) & (d) Dichlorovos; (e) & (f) Glyphosate. Residues involved in binding to compounds are showed as green lines and are labelled. H-bonds are highlighted as magenta dashed lines.

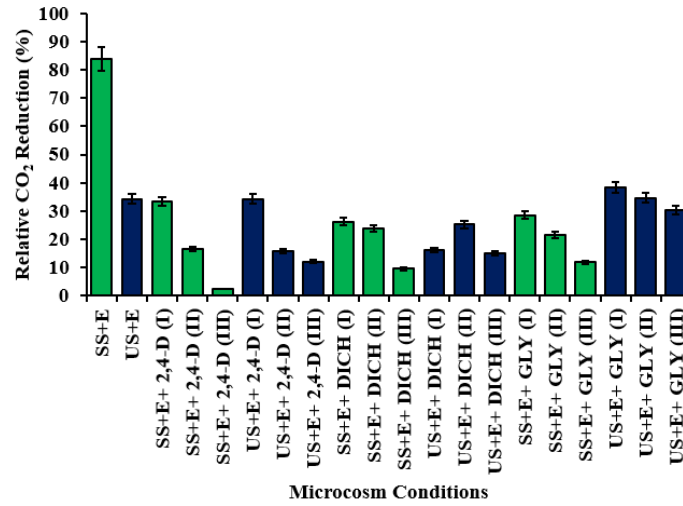


Fig 5: Effect of pesticides on CA mediated carbon dioxide reduction in soil microcosm.

Table 1: The Experimental conditions in the soil microcosm study for carbon sequestration inhibition using pesticides

Microcosm Conditions	Description
SS	Sterile soil
US	Unsterile soil
SS+E	Sterile soil with CA
US+E	Unsterile soil with CA
SS+E+2,4-D (I)	Sterile soil with CA along with different concentrations of 2,4-D
SS+E+2,4-D (II)	
SS+E+2,4-D (III)	
US+E+2,4-D (I)	Unsterile soil with CA along with different concentrations of 2,4-D
US+E+2,4-D (II)	
US+E+2,4-D (III)	
SS+E+DICH (I)	Sterile soil with CA along with different concentrations of Dichlorvos
SS+E+ DICH (II)	
SS+E+ DICH (III)	
US+E+ DICH (I)	Unsterile soil with CA along with different concentrations of Dichlorvos
US+E+ DICH (II)	
US+E+ DICH (III)	
SS+E+GLY (I)	Sterile soil with CA along with different concentrations of Glyphosate
SS+E+ GLY (II)	
SS+E+ GLY (III)	
US+E+ GLY (I)	Unsterile soil with CA along with different concentrations of Glyphosate
US+E+ GLY (II)	
US+E+ GLY (III)	

[CA-carbonic anhydrase enzyme from *Bacillus halodurans* isolate PO15; herbicide concentrations I- 1% (w/v); II- 3% and III- 5%]

Table 2: Inhibition Kinetics of carbonic anhydrase with the agrochemicals

Agrochemicals	Parameters	Concentration of Agro-chemicals		
		1%	3%	5%
2,4-D	Km (mM)	21.56	41.93	44.97
	Vmax	222.22	131.57	91.74
	Ki (mM)	14.10	18.57	24.82
Glyphosate	Km (mM)	13.08	37.25	40.02
	Vmax	204.08	181.82	163.93
	Ki (mM)	11.23	23.22	44.92
Dichlorvos	Km (mM)	16.53	22.15	39.45
	Vmax	232.55	178.57	153.85
	Ki (mM)	7.18	43.65	49.53

Table 3: The amino acid residues involved in the ligand enzyme interaction.

Ligand	Type of interaction	Interacting residues	Cdoc Score
2,4-D	Hydrogen bonding	Gly65,Gly95,Val 147,Ser150	88.064
Dichlorvos	Hydrogen bonding	Val67,Ser138,Ser148,Ser150	68.3316
Glyphosate	Hydrogen bonding	Ile168, Thr69,sSer146,(SH)Gly65,Val67, Gly95, val147,His149,Ser150	76.4368