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COMPUTATIONAL ANALYSIS OF BIODEGRADATION PATHWAYS FOR CHLORPYRYFOS USING EAWAG-BIOCATALYSIS/ BIODEGRADATION DATABASE PATHWAY PREDICTION SYSTEM

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ABSTRACT

Introduction: Computational tools like pathway prediction systems are in vogue to examine the extent of degradation and possible pathways for exploration of degradative abilities of diverse microorganisms. They also give an idea of the tentative end products of the biodegradation mechanism. In-silico approach is mainly used in designing the experiments for biodegradation leading to bioremediation. Chlorpyrifos is a toxic organophosphate insecticide whose degradation pathway has not been extensively studied. It forms more toxic 3, 5, 6-trichloropyridin-2-ol which causes cancer thus, making it necessary to study its possible biodegradation mechanism. **Methodology:** In this study, eMolecules database and EAWAG Biocatalysis/Biodegradation Database-Pathway Prediction System (EAWAG-BBD-PPS), formerly called University of Minnesota Biocatalysis/Biodegradation Database-Pathway Prediction System (UMBBD-PPS), have been used to predict the degradation pathways of O,O-Diethyl O-3,5,6-trichloro-2-pyridyl phosphothiorate (chlorpyrifos). Intermediate compounds have been named using ChemDraw Ultra 8.0.6. **Result:** The predicted pathway showed that aerobic transformation of chlorpyrifos leads to formation of more toxic compounds viz. 3,5,6-tri-chloro-pyridinol (TCP) and diethylthiophosphoric acid (DETP). Further, aerobic transformation was not easy. De-chlorination and ring cleavage of TCP, which occurred much later in the pathway formed 2-hydroxyacetate which was metabolized through KEGG. DETP eventually produced thiophosphate and ethylphosphate which are much simpler and non-toxic and can mineralize into ethanol. **Conclusion:** Hence, it is concluded that plausible pathways predicted by EAWAG BBD-PPS explain microbial degradation mechanism and can aid in designing pesticide biodegradation/bioremediation experiments. It can be used to efficiently design microbial consortium that can completely mineralize chlorpyrifos or any other xenobiotic, thereby eliminating it from the environment.

KEY WORDS: Biodegradation, Bioremediation, Computational analysis, Chlorpyrifos, In-silico, O, O-Diethyl O-3, 5, 6-trichloro-2-pyridyl phosphothiorate.

INTRODUCTION

Environmental concern owing to indiscriminate use of insecticides and their associated recalcitrance is not a novel issue. However their method of degradation is gaining rapid magnitude. This makes identification of degradation patterns, a crucial factor in environmental biotechnology. Conventionally, multi-tiered experiments are performed to identify all variables influencing degradation but the major drawback associated with it is the time factor [1]. Microbes are considered as the tools of nature that help overcome this problem of recalcitrance [2]. Selection of specific microorganisms for the degradation is the key factor which consumes moderate time and resources. This has led to the evolution of various pathway prediction systems (PPS) like EAWAG-BBD PPS. PPS, METACYC and Kyoto Encyclopedia of Genes and Genomes (KEGG), which not only elucidate step by step degradation mechanism but also aid in designing biodegradation experiments and toxicity predictions [3]. EAWAG-BBD (Swiss Federal Institute For Environmental Science and Technology -Biocatalysis/Biodegradation Database) was formerly called University of Minnesota Biocatalysis/Biodegradation Pathway Prediction System (UMBBD-PPS) until 1st July 2014 when the rights had been sold to EAWAG. PPS gives fair idea about chemical nature of contaminant, pathways involved in degradation and if possible, microorganisms accountable for such degradation. It emphasizes onto the undergoing structural changes leading to breakdown and also gives detailed information about the type of transformation along with similar reactions [4]. In this study, we have selected globally used chlorinated organo-thiophosphate insecticide, miticide and acaricide chlorpyrifos (O,O-Diethyl O-3,5,6-trichloro-2-pyridyl phosphothiorate). Its structure comprises of a pyridine ring to which three chlorine atoms and a side chain are attached. The side chain contains a sulphur and a phosphorus moiety. Eco-toxicological studies reveal that principal degradation product of chlorpyrifos is TCP, which is much toxic than its parent compound [5]. It is highly resistant to degradation since cleavage of the pyridine ring does not occur effortlessly and the presence of chlorine atoms make it worse. Unlike chlorpyrifos, TCP is highly water soluble, thus being a potent groundwater contaminant. Chlorine atoms not only hinder the cleavage of the pyridine ring, but their elimination is also very thorny for microbes since free chlorine radicals are noxious to them [6, 7]. Despite the fact that several studies on degradation of chlorpyrifos by some bacteria and fungi are available, still our knowledge about the degradation pathway is insufficient as chlorpyrifos degrading microbes tend to form TCP [8-15]. Very few are known to utilize TCP as the energy source with mineralization being a rare case scenario [6, 16-17].

In this work, we describe the evaluation of possible pathways of chlorpyrifos biodegradation using EAWAG-BBD Pathway Prediction System. The relevance of obtained pathway was studied by comparing the obtained pathway products with those found and published in research articles. Thus pathway predicted by EAWAG-BBD PPS will not only help us to understand biodegradation pathway of chlorpyrifos but can also aid in designing series of experiments for biodegradation studies and for identification of the major degradation product(s) of chlorpyrifos. It may also aid in tracing the most likely degradation pathway(s) of chlorpyrifos.

MATERIALS AND METHODS

PPS performs 3 main tasks, viz. SMARTS (SMiles Arbitrary Target Specification) matching, rule matching, and biotransformation using databases, hardware, software and chemical representations [18]. The compound whose biodegradation is to be predicted can be drawn using the Marvin Sketch applet or represented by SMILES (Simplified Molecular Input Line Entry System) strings. The predicted pathways are displayed by Marvin View applet. The SMARTS language is an extension of SMILES (Simplified Molecular Input Line Entry System) for creating patterns of organic functional groups [4].

Compound details of chlorpyrifos like molecular weight (350.586), molecular formula ($C_9H_{11}Cl_3NO_3PS$), source, structural formula, CAS number (2921-88-2), compound ID and SMILES[Clc1c(OP(=S)(OCC)OCC)nc(Cl)c(Cl)c1]were collected from eMolecules database. eMolecules database also contains all entries provided by NIST, Pub Chem and Drug Bank and resolves the duplicated/non-unique index numbers automatically[4]. There are two search features- Standard search feature that entertains queries related to names, substructures and suppliers and expert search feature that handles queries regarding molecular weight range, CAS numbers, suppliers, etc[4]. The eMolecules can be accessed by using the URL:

http://www.emolecules.com/ EAWAG-BBD PPS uses substructure searching, rule-base and atom to atom mapping to predict the microbial catabolic reactions. The functional groups in the compound are recognized by PPS and biotransformation rules are used to predict the transformations. These rules are based on reactions found in scientific literature and are found in EAWAG Biocatalysis/Biodegradation Database [4]. PPS can be accessed at the EAWAG-BBD Pathway Prediction page, which can be reached from the "Pathway Prediction" link on the EAWAG-BBD home page, or by using the URL: http://eawag-bbd.ethz.ch/. The PPS predicts biodegradation of the chosen compound. User can select the compound and the desired transformation, whether all transformations need to be seen or only more likely aerobic transformations. The compound can be entered either “Drawing the Structure and Generating SMILES” or “Entering SMILES Directly”. The PPS uses Chemaxon's MarvinSketch and MarvinView Java applets as plugins which are used when the user selects to draw the structure and generate smiles [4]. The default settings display aerobic transformations upto 6 levels, three or more carbon atom containing compounds and stop before level that contains more than 10 compounds. These options are changeable throughout the process. “Aerobic Likelihood” is the likelihood that the reaction will occur under aerobic conditions, exposed to air, in soil (moderate moisture) or water, at neutral pH, 25°C, with no competing or toxic other compounds. At least two or more biodegradation experts assign the rules to aerobic likelihood More information on rule priority is available at, http://eawag-bbd.ethz.ch/servlets/pageservlet?ptype=allrules. The aerobic likelihood of a transformation is indicated by a color of an arrow that starts from one substrate to one or two products. Users can choose to view aerobic or all transformations of the desired compound. When aerobic transformation is selected but there is no very likely, likely or neutral transformation, then the setting is automatically changed to “All” and the resulting transformations are shown. SMILES retrieved for Chlorpyrifos was used in EAWAG-BBD PPS to predict the biodegradation pathway. PPS was accessed from http://eawag-bbd.ethz.ch/. SMILES of Chlorpyrifos compound were entered directly in EAWAG-BBD-PPS. First, all the possible products of chlorpyrifos degradation were predicted. Secondly, aerobic transformation of chlorpyrifos degradation was seen then detailed pathway for TCP was obtained. The intermediate compounds were named using ChemDraw Ultra 8.0.6.

RESULTS AND DISCUSSION

Chlorpyrifos Degradation: EAWAG-BBD PPS generated a reaction network involving possible pathways describing the biodegradation of chlorpyrifos. Figure1 represents all the possible degradation products of chlorpyrifos obtained by all the possible transformations (both aerobic and unlikely aerobic). Formation of 6 probable products, each with a separate degradation pathway was observed. The products were, TCP, DETP, O,O-diethyl O-3,5,6-trichloropyridine-2-yl phosphothiorate, O-3,5-dichloropyridin-2-yl O,O-diethyl phosphothiorate, O-3,6-dichloropyridin-2-yl O,O-diethyl phosphothiorate and O-5,6-dichloropyridin-2-yl O,O-diethyl phosphothiorate.

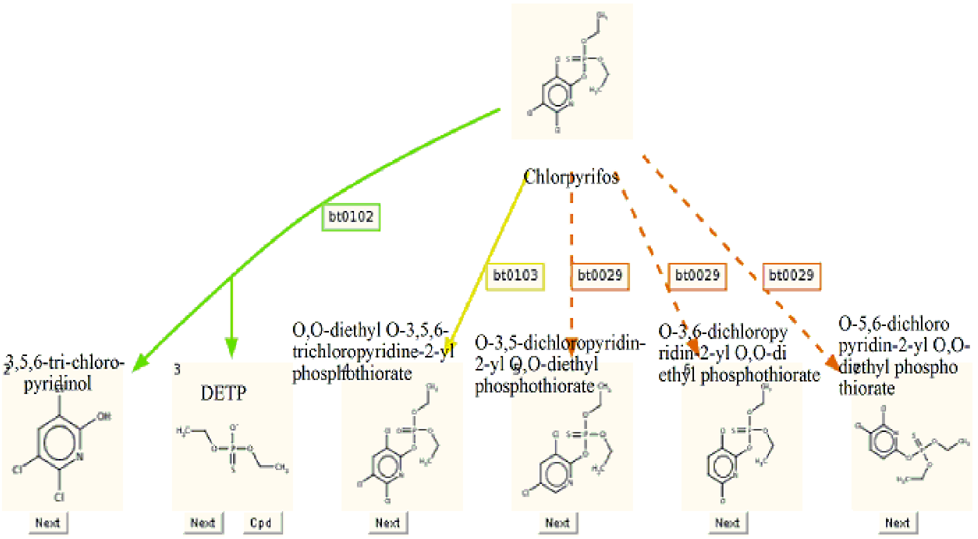

Fig 1: Chlorpyrifos Degradation Pathway Prediction Results From EAWAG-BBD PPS.

The most likely aerobic transformation shown by In-silico analysis was breakdown of chlorpyrifos into TCP and DETP. When observed closely, it was noticed that conversion of chlorpyrifos into TCP removed the side chain of the parent compound but all 3 chlorine atoms remained intact. The broken chain from chlorpyrifos resulted in the formation of DETP. Similar results have been shown by *Enterobacter* sp., *Pseudomonas desmolyticum*, where TCP and DETP are the major products [17, 19]. Aerobically neutral transformation yielded O,O-diethyl O-3,5,6-trichloropyridine-2-yl phosphothiorate ($C_9H_{11}Cl_3NO_4P$) with a removal of sulphur. This transformation resulted due to the conversion of thiophosphate-diester into phosphatediester (rule bt0103). Aerobically unlikely transformation can yield 3 products as a result of transformation of organohalide into RH (rule bt0029). Thus, O-3, 5-dichloropyridin-2-yl O, O-diethyl phosphothiorate, O-3, 6-dichloropyridin-2-yl O,O-diethyl phosphothiorate and O-5,6-dichloropyridin-2-yl O,O-diethyl phosphothiorate formation reduced one chlorine atom but in this case S and P moiety remain intact. Position of chlorine atoms was the only difference between these three products. Although aerobically unlikely, but this reaction removed a chlorine atom from the compound, thereby reducing its overall toxicity. Since it is unlikely, we do not have any reports citing these three as degradation products. Possibility of photo-catalytic, physical or environmental degradation leading to their formation cannot be ignored.

DETP Degradation: It is known that existence of chlorine inhibits degradation but it is interesting to note that among the two major degradation products, DETP and TCP, latter is the major contaminant due to high water solubility. The broken chain from chlorpyrifos resulted in the formation of DETP, as a result of conversion of aromatic thiophosphate to hydroxyl-aromatic derivative and thiophosphate derivative, while aromatic amidothiophosphate was converted to hydroxyl-aromatic derivative and amidothiophosphate (rule bt102). Further, DETP ($C_4H_{10}O_3PS^-$) being a much simpler compound can yield 3 different products with the application of 2 biodegradation rules. With the removal of alkyl group and hydroxyl ion, DETP was broken down into ethyl thiophosphate and ethanol (rule bt0361). This rule states that aliphatic phosphodiester is converted to alcohol and aliphatic phosphoester. Further with the same rule, ethylthiophosphate was converted to thiophosphate with the removal of the only alkyl group left. Ethyl thiophosphate can also transform into ethylphosphate if thiophosphate is converted to phosphate with the removal of sulphur from the molecule (rule bt0103). The third transformation of DETP was into diethylphosphoric acid, with the removal of sulphur moiety, since aryl phosphoester was converted to phenol derivative, aryl phosphodiester into phenol derivative and phosphoester, while aryl phosphotriester forms phenol derivative and phosphodiester (rule bt0103). This reaction is reversible and handles the enol-keto tautomerization. Diethyl phosphoric acid is structurally similar to DETP except the release of sulphur moiety. Diethyl phosphoric acid then transformed into ethylphosphate with removal of one alkyl group (rule bt0361). Thiophosphate and ethylphosphate, the simplest and non-toxic products of DETP biodegradation tend to mineralize into ethanol. Thus, DETP can be utilized as sulphur source by *Pseudomonas acidovorans* and phosphorus source [9]. In the above case, DETP does not work as carbon source but has the potential to be used as sulphur and phosphorus source. This is evident by the literature as chlopyrifos is utilized as S and P source by various species of *Aspergillus*, *Penicillium brevicompactum* and *Trichoderma harzianum* [20]. In some rare events, it was found that organisms like *Enterobacter* sp. can metabolize chlorpyrifos into TCP and DETP and utilized the former as both carbon and phosphorus source [19, 21] while Shelton [22] reported no degradation while working with microbial consortium from cattle dip. When DETP was not utilized as S source, it was not degraded any further and accumulated in the culture medium [11-15]. This explains as to why microbial consortium utilizing DETP as carbon source was unable to degrade it in the presence of P and S source [22].

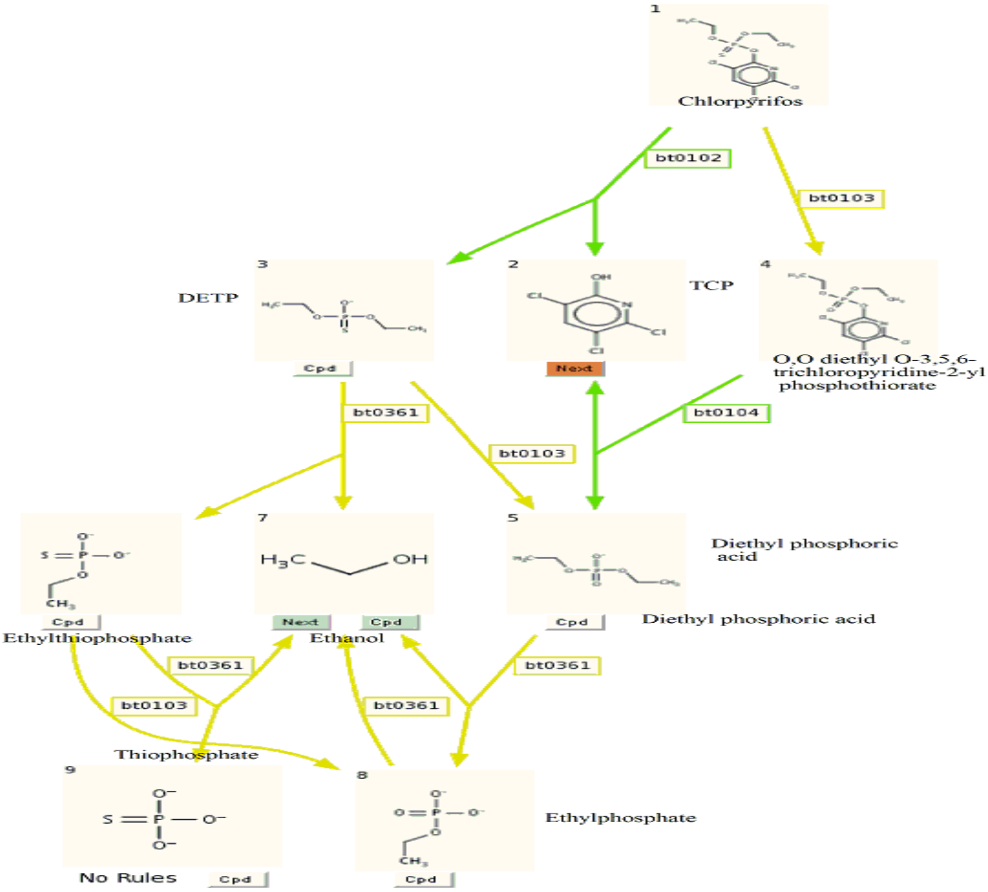

Fig 2: DETP degradation pathway prediction results from EAWAG-BBD PPS.

TCP Degradation

Conversion of chlorpyrifos into TCP removed the side chain of the parent compound but all 3 chlorine atoms remain attached to the pyridinol ring. Cleavage of the ring was possible only after the removal of the chlorine moieties, which will thereby reduce its overall toxicity. The predicted pathway for TCP degradation involved complex tracks. First step was the removal of a chlorine moiety with the formation of 3, 5-dichloropyridin-2-ol or 3,6-dichloropyridin-2-ol, depending on the position of chlorine atoms. Although aerobically unlikely, this reaction followed the conversion of organohalide into RH (rule bt0029). This reductive dehalogenation removes one chlorine atom and opened the path for further dechlorination, leading to formation of 3-chloropyridin-2-ol with single chlorine present at meta- position (rule bt0029). Removal of third chlorine did not take place, instead two hydroxyl ions attached to 3-chloropyridin-2-ol and form 5-chloropyridin-2, 3, 6-triol. These reactions prove that dechlorination occurs in sequential manner. This transformation was done by rule bt0005* which is a combination of different rules viz. bt0367 and bt0255. It represented dioxygenation of *vic*-unsubstituted aromatics and their subsequent oxidation to form the catechol derivative. When more than one potential site is present, dioxygenation position is determined by orientation(s) of the ring(s) in the enzyme's active site, which cannot be predicted. This explains to why TCP degradation is rarely reported, since free chlorine is toxic to microorganisms, thus making TCP metabolism difficult [7]. Formation of 5-chloropyridin-2,3,6-triol helps in breaking of the pyridinol ring of TCP and formation of (2E,4E)-3-chloro-4-formyl-4-hydroxybut-2-enoate as a result of intra-diol ring cleavage of *vic*-dihydroxypyridine (rule bt0254). *Pseudomonas* sp. mineralized TCP in liquid medium by reductive dechlorination pathway [6, 16]. In this pathway, TCP was reductively dechlorinated twice and yielded chlorodihydroxy-2-pyridone then tetra-hydro-2-pyridone. Ring cleavage formed maleamide semialdehyde which degraded into water, carbon dioxide and ammonium ions. (2E, 4E)-3-chloro-4-formyl-4-hydroxybut-2-enoate was further broken down into (E)-4-amino-3-chloro-4-oxobut-2-enoate as N-carboxylate amide forms amine with the release of carbon-di-oxide (rule bt0280). It was interesting to note that enamine form of the amide N-carboxylate first tautomerized. Further primary amide transformed into carboxylate with the removal of $-NH_2$ (rule bt0027) and lead to the formation of 2-chloromaleate. Surprisingly this reaction was highly aerobic. A study on hydrolysis of chlorpyrifos by *Phanerochaete chryrysosporium* causing the cleavage of pyridinyl ring and formation of carbon dioxide and water has been reported [23]. Silambarasan and Abraham [24] have reported hydrolysis of TCP by *Alcaligenes* sp. JAS1. Thus, if somehow a single microbe or consortium is able to utilize TCP as energy source and cause the cleavage of the pyridinol ring, the structure resulting from the broken ring can be easily utilized by aerobic microbes and can lead to the formation of much simpler products and can even lead to mineralization of TCP. The broken pyridine ring of 2-chloromaleate, further transformed into 2-chloro-3-hydroxysuccinate (rule bt0021). 1-Oxo 2- or 3-ene derivative is converted to 1-Oxo-3-hydroxy derivative; this rule handles the hydration of activated double bonds alpha or beta to the oxo group found in thioesters, carboxylates and ketones. Removal of H^+ from 2-chloro-3-hydroxysuccinate formed 2-choloro-3-oxosuccinate as a result of conversion of secondary alcohol into ester/ketone (rule bt0002). Transformation of 2-ketocarboxylate into carboxylate with the removal of CO_2 led to the formation of 2-chloromalonate (rule bt0082). After decarboxylation, some 2-ketocarboxylates transiently form an aldehyde, but this is quickly oxidized to the carboxylate. Further 2-chloromalonate can be easily degraded into 2-chloroacetate, as 2- or 3- substituted Carboxylate forms alkyl halide (RH) with the release of CO_2 (rule bt0051). This rule decarboxylates most 2-amino, 2-aryl, 2-tertiary carbon, 2-N-heterocyclic aromatic, 3-keto, 3-ene-5-keto, 3-hydroxy, and 3-aryl carboxylates. Similarly 2-chloroacetate was degraded into 2-hydroxyacetate by hydrolytic dehalogenation of aliphatic halomethyl and dihalomethyl derivatives (rule bt0022). Halomethyl derivative forms 1-Methylalcohol derivative while Dihalomethyl derivative tends to form 1-Halo-1-methylalcohol derivative. Surprisingly the last chlorine remained attached till the formation of 2-chloroacetate and was finally released when 2-chloroacetate degraded to form 2-hydroxyacetate. Formation of 2-hydroxyacetate was definitely not a mineralization reaction but it was further metabolized by KEGG. This explains complete mineralization of chlorpyrifos by *Serratia* sp. and *Trichosporon* sp. [25]. Co-culture of *Cellulomonas fimi* degraded chlorpyrifos into TCP and *Phanerochaete chrysosporium* utilizes TCP as energy source [26]. Both these studies indicated

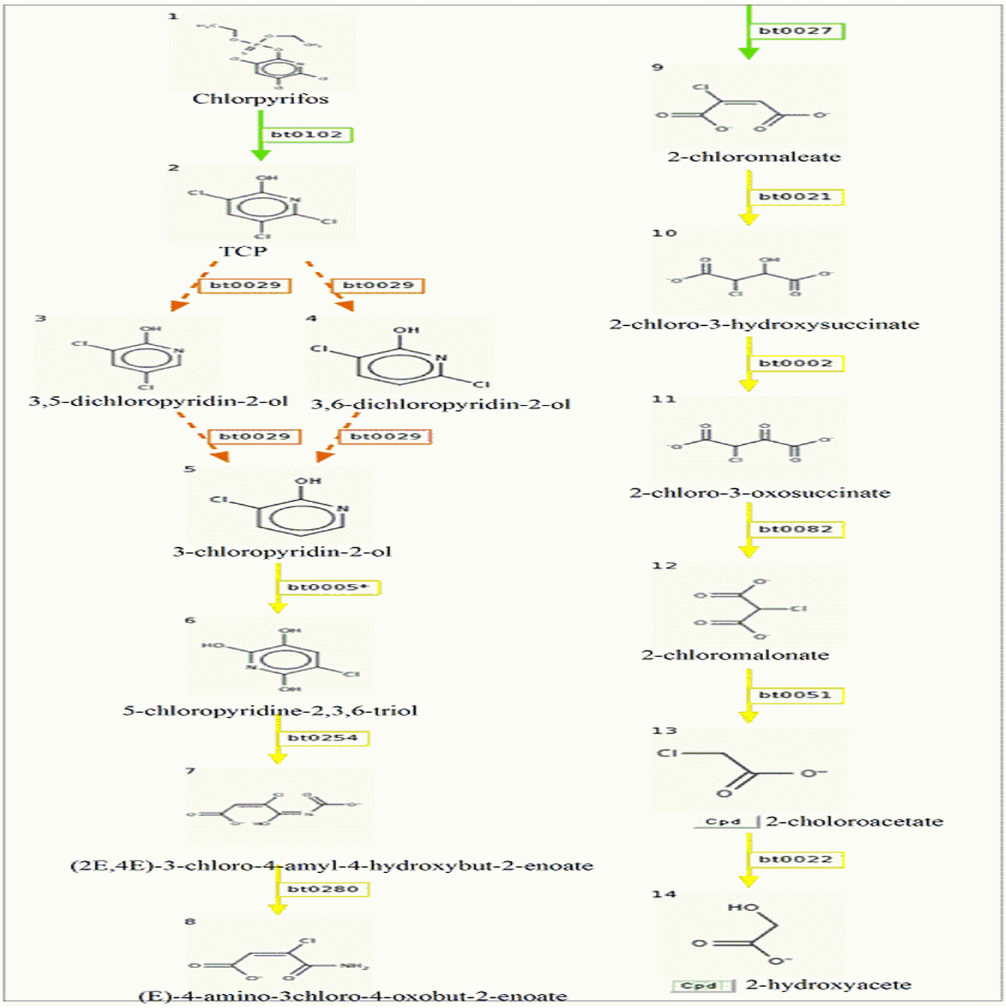

Fig 3: TCP degradation Pathway prediction results from EAWAG-BBD PPS.

That mutualism or co-metabolism can be implied to achieve complete degradation of chlorpyrifos since single microorganism is most of the times is insufficient.

CONCLUSION

In the present study, EAWAG-BBD PPS devised the step-wise mechanism and possible transformations of chlorpyrifos. Plausible pathway suggested the breaking of chlorpyrifos into TCP and DETP which is supported by several studies. Thiophosphate and ethylphosphate were predicted end products of chlorpyrifos which tend to form ethanol. TCP degradation is a difficult method since most reactions were unlikely aerobic and removal of chlorine atoms is necessary. Obtained pathway elucidated that successive dechlorination removes 2 chlorine atoms out of 3 from TCP and then pyridinol is broken. After a series of steps, it tends to form 2-hydroxyacetate, which is metabolized through KEGG. Thus, it helps in understanding microbial degradation mechanism by predicting biodegradation pathway with all the possible intermediate products. EAWAG-BBD PPS has proven its worth as useful tool in designing biodegradation experiments and can aid in the identification of end products.

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