MATERIALS AND METHODS
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3.1 Soil sample Collection:

Collection of soil samples was done from grape wine yards from different locations of Sangli district, M.S. India, having known history of organophosphorus insecticides use since at least last five years. The soil samples were collected from Walwa (Walwa Tehsil), Bhisur (Miraj Tehsil), Palus (Palus Tehsil), Vita (Khanapur Tehsil), Miraj (Miraj Tehsil), Takari (Walwa Tehsil) and Tasgaon (Tasgaon Tehsil). These regions were selected specifically because; out of 10 Tehsils of Sangli district above Tehsils are highest grape producing regions. These areas are located to South region of Maharashtra (India). In the present research work, they termed as regions for the convenience of description of the isolates. A total of 25 soil samples from different grape wine yards have been collected and screened for insecticide resistant / degrading bacteria. The soil was collected from about 10-12 cm depth of rhizosphere region, plant material was discarded manually and soil sample was sieved. They were brought to the laboratory further in polyethylene bags and kept at 4°C, until analysis was conducted.

Figure No.4: Map of Sangli District, showing all the localities from where samples of soil were collected

3.2.1 Media Used:
The different media viz. mineral salts medium and Nutrient Agar were used during the present study. Mineral Salts Medium (MSM) a liquid preparation was prepared by adding 0.3gm NaNO$_3$, 0.05gm MgSO$_4$, 0.001gm FeSO$_4$, 0.1gm K$_2$HPO$_4$, 0.05gm KCl, 0.5gm KH$_2$PO$_4$, 0.05gm Yeast Extract and 1.0gm glucose in 100ml D/W. Nutrient agar was prepared by adding 0.25% Meat Extract, 1.0% peptone, 0.5%. Sodium chloride and 2.5% Agar, for liquid medium preparation (MSM), agar was not added to the above medium. The pH of media is between 7.0-7.2 for both media.

3.2.2 Sterilization of Media, Solutions and Apparatus: Media were sterilized throughout the study by autoclaving at 121$^\circ$C, 15 Lbs pressure for 20 minutes. Glass apparatus were sterilized in an oven at 180$^\circ$C for 20 minutes.

3.3 Insecticides Used: In the present study, technical grade of two organophosphorus insecticides viz. Quinalphos (25% E.C.) and Dichlorvas (76% E.C.) were used. These insecticides were obtained from local market of sangli, M.S. Maharashtra.

3.3.1. Quinalphos:

An organothiophosphate insecticide Quinalphos is extensively used for controlling insect pests of orders Coleopteran, Dipterans, Lepidoptera, Hemiptera on vegetables, fruit trees, cotton and rice in agriculture.

Table no.4: Identification, classification and properties of Quinalphos

Identification (Source: EPA, EU)
<table>
<thead>
<tr>
<th>ID</th>
<th>239</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>quinalphos</td>
</tr>
<tr>
<td>CAS</td>
<td>13593-03-8</td>
</tr>
<tr>
<td>IUPAC</td>
<td>o, o-diethyl, o-quinoxalin-2-yl-phosphorothioate</td>
</tr>
<tr>
<td>Formula</td>
<td>$\text{C}<em>{12}\text{H}</em>{15}\text{N}<em>{2}\text{O}</em>{3}\text{PS}$</td>
</tr>
</tbody>
</table>

**Classification**

<table>
<thead>
<tr>
<th>Mode of Action</th>
<th>Contact and stomach action. Acetylcholinesterase (AChE) inhibitor.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pesticide Type</td>
<td>Insecticide, Acaricide</td>
</tr>
<tr>
<td>Chemical Group</td>
<td>Organophosphate</td>
</tr>
</tbody>
</table>
| Classification | Heterocyclic organothiophosphate insecticides  
Quinoxaline organothiophosphate insecticides  
Organothiophosphate acaricides  
Organothiophosphate insecticides |

**Properties (Source: PubChem)**

<table>
<thead>
<tr>
<th>Molecular Weight</th>
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</thead>
<tbody>
<tr>
<td>Physical State</td>
<td>Colorless crystals</td>
</tr>
<tr>
<td>Surface Area</td>
<td>280.97</td>
</tr>
<tr>
<td>Polar Surface Area</td>
<td>95.37</td>
</tr>
</tbody>
</table>

**Structure**
3.3.2. Dichlorvas:

It is an organophosphate used against caterpillars, aphids, thrips, whiteflies and spider mites, mushroom flies, in outdoor fruit, vegetable crops and greenhouse. It acts through contact as well as a stomach poison against various insects.

Table no.5: Identification, classification and properties of Dichlorvas

Identification (Source: EPA, EU)

<table>
<thead>
<tr>
<th>ID</th>
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<tbody>
<tr>
<td>Name</td>
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</tr>
<tr>
<td>CAS</td>
<td>62-73-7</td>
</tr>
<tr>
<td>IUPAC</td>
<td>2,2-dichlorovinyl dimethyl phosphate</td>
</tr>
<tr>
<td>Formula</td>
<td>C₄H₇Cl₂O₄P</td>
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</tbody>
</table>

Classification:

<table>
<thead>
<tr>
<th>Mode of Action</th>
<th>Respiratory, contact and stomach action. Acetylcholinesterase (AChE) inhibitor.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pesticide Type</td>
<td>Insecticide, Acaricide, Metabolite</td>
</tr>
<tr>
<td>Chemical Group</td>
<td>Organophosphate</td>
</tr>
<tr>
<td>Classification</td>
<td>organophosphate insecticides organophosphate acaricides</td>
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</table>
Properties (Source: PubChem)

<table>
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<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
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<tr>
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<tr>
<td>AlogP</td>
<td>0.473</td>
</tr>
<tr>
<td>Surface Area</td>
<td>196.89</td>
</tr>
<tr>
<td>Polar Surface Area</td>
<td>54.57</td>
</tr>
</tbody>
</table>

Structure:

![Structure of Dichlorvas](image)

Figure No.6: Structure of Dichlorvas

3.4 Enrichment of soil samples:

The bacterial strains capable of degrading organophosphorus insecticides as Quinalphos and Dichlorvas were isolated from grape wine yard soils by technique as Enrichment Culture Technique. This technique involves Mineral Salts Medium (MSM) with variable concentrations of insecticide. In 500 ml capacity Erlenmeyer flask, collected soil sample (1g) was added in 100 ml mineral salts medium. Incubation was done for 7 days at 25-30°C on shaker at 200 rpm. The insecticides Quinalphos and Dichlorvas were used in the concentrations in mg/L as 5, 10, 15 and 20 in mineral salts medium for enrichment of soils.

3.5 Isolation and maintenance of insecticide resistant bacterial strains
During enrichment, one loop full enriched culture was streaked on Nutrient Agar plates added with same concentration of insecticide at 24 hrs intervals until pure cultures were isolated. On nutrient agar plates, single, pure, isolated bacterial colonies which differ morphologically were subcultured containing same concentration of insecticides. The isolated pure strains were maintained and preserved at 4°C and also subcultured after every two months.

3.5.1 Purification of isolated strains:

An individual isolated colony of insecticide degrading bacteria was taken up with the help of sterilized wire loop and streaked on nutrient Agar medium. Each isolated strain was streaked at least three to four times on nutrient agar plates for purification. After the purified isolates were obtained, they were restreaked on agar medium containing respective insecticide for confirmation of the isolate. The pure bacterial strains were characterised depending on the classification given in Manual of Bergey’s on Systematic Bacteriology.

3.5.2 Physical and biochemical characterization of isolates:

a. Colony morphology-

Colony morphology of each isolate was studied as size, shape, color, margin, elevation and opacity.

b. Gram staining:

Gram staining technique was used for differentiation between Gram positive and Gram negative bacterial strains (Benson, 1994).

c. Growth on Mac- Conkey’s Agar:

On Mac-Conkey’s agar plate, single colony of each isolate was streaked and incubation was done at room temperature for about 24 hrs. (Chessbrough, 1993). Appearance of growth in the form of pink colored colonies after 24 hours incubation indicates lactose fermenting capacity of the isolate.

d. Catalase Test:
A thick growth of the test isolate was immersed in 3ml of 3% H$_2$O$_2$ solution with the help of a sterile wire loop. Active bubble formation within a few seconds showed a positive catalase activity (Benson, 1994).

e. Oxidase Test:

A filter paper strip was soaked with the freshly prepared oxidase reagent. (1.0g tetra methyl-p-phenylenediamine dihydrochloride in 100 ml distilled water). Using a sterile wire loop, a colony of the isolate was rubbed on the above filter paper & the paper was observed for any change in the color of the reagent (Benson, 1994).

f. Coagulase Test:

On a clean slide, two drops of saline solution (0.85%) were taken and the test culture was smeared on one of them. Then a single drop of human blood plasma was added to both the drops and the drops were observed under a dissecting microscope for rapid agglutination of plasma (Cheesbrough, 1993).

g. Urease test:

The Christensen’s urea agar slants (0.1g peptone, 0.2g K$_2$HPO$_4$, 0.5g sodium chloride, 0.6ml phenol red were. Added in 150ml distilled water, pH 6.8 – 6.9 then 2g agar was added and the medium was autoclaved. After sterilization 20% urea was added to medium. It was readily added into sterilized test tubes to make slants. The slants were inoculated with the test isolate and incubated at room temperature for 24hrs (Benson, 1994). Appearance of pink color showed positive test.

h. Starch hydrolysis test:

The test organisms were streaked on sterile starch agar plates (2.0g soluble starch, 0.3g Beef extract, 0.5g peptone, in D/W 100ml, pH adjusted to 7.0 and 2.5g agar) and incubation was done for 24 hrs at room temperature. After incubation gram’s iodine solution was poured on these plates and plates were kept for one hour at room temperature (Benson, 1994). Observation of clear area around the growth showed positive result, while blue color indicated that starch is not hydrolyzed.
i. Sporulation test:

The bacterial isolates were grown at room temperature on Nutrient agar medium for one week. The smear was prepared from the culture, air dried and fixed by heating by passing through flame. The smear was covered with malachite green (5%). Solution for 3-4 minutes by heating the slide with continuous steering. Slide was washed and saffranine was applied for 1-2 minutes. Slide was washed, air dried and observed under oil immersion lens. The presence of green colored spherical or oval bodies indicated a positive result.

j. Methyl red test:

The medium was prepared by dissolving 0.5g peptone and 0.5g K2HPO4 in 100 ml D/W, PH adjusted at 7.6 and medium poured in test tubes. The medium was autoclaved at 121°c for 20 minutes. Glucose solution, 0.25ml was added in 5 ml medium in each tube. The above medium at room temperature for 24 hrs. Then after incubation 5 drops of methyl red (prepared by dissolving 0.1g methyl red, 300 ml ethanol in 100ml distilled water) were added and mixed. The bright red color indicated the positive test (Cheesbrough, 1993).

k. Citrate utilization:

The Simmon’s citrate medium was prepared (NaCl 0.5g, NH4H2 PO4 0.19, MgSO4 7H2O 0.02g, 0.2% Bromothymol blue 4ml, poured in test tubes (5ml each), autoclaved) and inoculated with respective isolated and incubated at room temperature (Benson, 1994). The color change from green to blue indicates positive result.

l. Nitrate reduction:

The peptone nitrate broth was prepared by dissolving peptone. 2.0gm, potassium nitrate 0.19 Disodium phosphate 0.29, Dextrose 0.01g, pH adjusted to 7.2 and agar 2.0g in 100ml Distilled water. The medium was autoclaved after adding 5ml medium in each test tube. The medium was added with the fresh culture suspension of each isolate and incubation was done at room temperature for 24 hrs (Benson, 1994).

m. Voges-Prauskaur (V.P) test:
The medium for V.P. Test was prepared by adding 0.5 g peptone 0.5 g di-potassium hydrogen phosphate in 100 ml distilled water. 0.5 g of dextrose was added and pH adjusted to 7.6. Medium was inoculated with respective isolated and incubated at room temperature for 24 hrs. After incubation, 1 ml 40% KOH and 3 ml 5% naphthol were added. Contents were properly shaken and checked for the appearance of red color.

n. Indole test:

The Indole test medium was prepared by dissolving 2 g peptone and 0.5 g sodium chloride in 100 ml D/W, pH-7.4. This medium was poured in test tubes and autoclaved. Inoculation of bacterial isolate in sterile medium and incubation of this medium was done at room temperature for 24 hrs. After incubation 0.5 ml of Kovac’s reagent (10 g p-dimethylaminobenzaldehyde, 150 ml isoamyl alcohol and 50 ml conc. HCl) was added and shaken gently. The change in color was observed, development of red color in the upper layer showed positive result.

o. Motility test:

The motility of each isolate was observed by hanging drop technique. One drop of suspension was taken on a coverslip and with the help of wax coverslip was placed on a slide in inclined position so as to drop hang over. Then the motility was observed in low power and high power lenses.

p. H₂S production test:

The medium for H₂S production test was prepared by dissolving 2.0 g peptone and 0.5 g sodium chloride in 100 ml D/W, pH adjusted to 7.2. In 10 ml capacity tubes this medium (5-6 ml each) was distributed, sterilised and added with respective isolate. Tubes were kept for incubation for 24 hrs at room temperature, with filter paper strip inserted in each tube. After incubation, positive test was indicated by blackening of filter papers.

q. Acid Release from Sugars (Glucose, Lactose, Sucrose and Mannitol)

The test is used to differentiate organisms that ferment a specific sugar with acid and a gas production. The based medium was prepared by dissolving 1 g peptone and 0.5 g sodium
chloride in 100ml distilled water and 0.25ml 1% Andrade’s indicator (0.5% acid fuchsin and add 1N NaOH till colour turns slight Yellow). Then the medium is distributed in different tubes, 5ml each. These tubes were autoclaved. On cooling filter sterilized respective sugar (Glucose, Lactose, Sucrose and Mannitol) added into basal medium. The fresh culture of respective bacterial isolate was inoculated into each tube. Incubation of these tubes was done for 24 hrs at room temperature. Appearance of pink colour indicated acid production and gas production is revealed by the formation of a bubble in the inverted Durham’s tube (Benson, 1994).

3.6 Degradation of Quinalphos and Dichlorvas by selected isolated bacterial strains:

About 25 bacterial isolates have been isolated from collected soil samples. So depending on the tolerance limit of all the 25 isolates, we have selected only 6 isolates for the study of degradation of insecticides. The selected strains for the degradation pattern study were WL Dump Q10, WL Dump D10, WL Dump D2O, TK Q20, VTQ15 and PLD15.

To study degradation of Quinalphos and Dichlorvas the isolated strains were inoculated in the MSM broth with the respective concentrations of insecticide to which they resist. Out of 6 isolates, three isolates were Quinalphos resistant and remaining three isolates were Dichlorvas resistant. The concentrations of respective insecticide that is Quinalphos were selected as WL Dump Q10 (10mg/L), VTQ15 (15mg/L), TKQ20 (20mg/L), and Dichlorvas as WL Dump D10 (10mg/L), WL Dump D20 (20mg/L) and PL D15 (15mg/L).

Thus, for each isolate 100ml of mineral salts medium supplemented with respective insecticide concentration was used. Then after sterilization medium was inoculated with an isolate and on rotary shaker at 150 rpm, incubation was done at room temperature. Five milliliter of broth was taken from above medium for detection of contents of flasks and for 10 min centrifuged at 5000 rpm. Supernatant was used for analysis by UV-visible spectrophotometer to detect degradation of insecticide by selected isolate and remaining pellet was discarded. Degradation was detected conducted for interval of every 2 days for 8 days. The degradation rate of each isolate was also determined.

3.7 Determination of optimum Growth conditions for Degradation:
For optimizing growth conditions of isolates, two parameters as temperature and pH were considered for each isolate and for this following methodology were adopted.

3.7.1 Determination of Optimum Temperature:

To detect the optimal growth conditions for degradation of insecticides, single parameter test was carried out in this study under different conditions for this purpose 5ml of mineral salt medium was added in 6 sets, each of six test tubes for each isolate and autoclaved. Also the medium was supplemented with 50ml of freshly prepared isolate culture. Six sets of tubes were incubated at 10°C, 20°C, 25°C, 30°C, 35°C, and 40°C for 8 days. After incubation the percent degradation of an insecticide were determined by following formula,

\[
\text{Percent degradation} = \frac{\text{Ab} - \text{Aa}}{\text{Ab}} \times 100
\]

Where,

\( \text{Aa} \) = absorbance of compound after degradation

\( \text{Ab} \) = absorbance of compound before degradation at the same wavelength.

The absorbance was taken at 225 nm for Quinalphos acid at 220nm for Dichlorvas and then graph was plotted between temperature along X-axis and percent degradation along the Y-axis. This graph provided information about optimum temperature of the test organism.

3.7.2 Determination of optimum pH:

To determine the optimum pH, the mineral salt medium, 5 ml was added in 6 sets, each of 6 test tubes, for each isolate and their pH were 4.0, 5.0, 6.0, 7.0, 8.0, & 9.0. These test tubes were autoclaved and then inoculated with 50ml of fresh culture of an isolate. Also the medium of each test tube was supplemented with respective insecticide concentration. The cultures were incubated for 6-8 days at their optimum temperature (which allowed these cultures to grow). After incubation the percent degradation of insecticide by isolate was determined by same formula. The graph was plotted between pH on X-axis and percent degradation on Y-axis and percent degradation along Y-axis. This graph provided the value of optimum pH for the test organism.
3.8 Extraction of the insecticides for residue analysis and detection of metabolites of Quinalphos and Dichlorvas:

Centrifugation of Degraded broth was done at 10000 rpm for 15 min after 5 days incubation. After centrifugation, supernatant was taken for extraction of metabolites with ethyl alcohol (1:1). Evaporation of extracts was done in an evaporator till dryness. GCMS analysis was done by dissolving obtained residue in methanol in small volume and used for. Then, gas chromatography attached mass spectroscopy was used for analysis of extract. This chromatography was performed in a specific programming mode along temperature with DB5MS 30m capillary column (0.25 column ID and 2.5 micron particle size). Then sample was injected into temperature program with a split mode of 180°C for 1.5 min, 250°C for 10 min, at the normal rate of 10°C/min, temperature of injector was 250°C for 15 min at a rate of 10°C/min, injector temperature was 250°C and temperature of detector was 250°C. Carrier gas used was Nitrogen. Components were analyzed and compared with NIST library.
3.9 16s rRNA sequencing and phylogenetic relationship

An efficient molecular technique was discovered with quantitative mode for rapid analysis of diversity of microbial community in soil. Following method was carried out for 16s rRNA sequencing.

3.9.1 Identification of a Microbial Culture using 16S rRNA based Molecular Technique:

Experimental Method:

The method used for 16s rRNA sequencing for all the five isolates is same and is as follows:

1. Isolation of DNA was done from given culture of each isolate and 1.2% Agarose Gel was used for evaluation of DNA quality and it has been observed a single band on gel which is of high-molecular weight DNA.
2. Amplification by Polymerase Chain Reaction technique was carried out and from isolated DNA a 16S rDNA gene fragment was obtained. When loaded and ran on Gel a single defined band of 1500 bp Polymerase Chain Reaction amplicon was observed (Gel Images).
3. Contaminants were removed to purify PCR amplicon.
4. DNA sequencing of PCR amplicon was carried out in forward and reverse sequencing by using BDT v3.1 Cycle sequencing kit, with 8F and 1492R primers on ABI 3730xl Genetic Analyzer.
5. Using aligner software, generation of consensus sequences of 16S rDNA gene was done for each isolate from reverse and forward sequence data. Consensus sequences generated for five isolates WL DumpQ10, VT Q15, WL DumpDQ20, WL DumpD10 and PL D15 as 1367 bp, 1370 bp, 1424 bp, 1446 bp and 1352 bp respectively.
6. By using sequence of 16S rDNA gene, BLAST was carried out with the nr database of NCBI genbank. First ten sequences were selected based on maximum identity score and Clustal W software program was used for alignment using multiple alignments. By using RDP database distance matrix was generated and by using MEGA 4 phylogenetic tree was constructed.
3.9.2 Phylogenetic Analysis and Identification of Selected Strains:

For full length 16s rRNA gene sequences selection genbank nucleotide sequence database was used for each representative strain. For alignment of sequences which were determined in this work, sequence alignment software, Clustal W was used with the representative sequences (Thomson et.al., 1997). Neighbor-Joining method was used by which evolutionary history was inferred (Saitou and Nei, 1987). The consensus tree with bootstrap was taken which was taken from 500 replicates to represent evolutionary history of analyzed taxa (Felsenstein, 1985). By using Kimura 2-parameter method evolutionary distances were computed (Kimura, 1980).

3.9.3 Distance Matrix Method:

This method requires multiple sequence alignment (MSA) as an input, because phylogenetic analysis specifically depends on measurement of "genetic distance" between the classifying sequences. This method attempts to prepare an all-to-all matrix by using sequence query set giving distance within each sequence pair. With obtained information construction of a phylogenetic tree was done that represent closely interrelated sequences under the same interior node and their branch lengths closely reproduce observed distances between their sequences.