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**Original Article**

# Biodegradation Study of an Organophosphorus Insecticide–Quinalphos by Novel *Sphingobacterium mizutaii* strain DSM 11724

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## Abstract

Organophosphate pesticides are now the most popular commercial pesticides in vineyards, with widespread use across the globe. They are recalcitrant, have acute toxicity at higher concentrations and irreversibly inactive acetylcholine esterase in insects, humans and other animals. *Sphingobacterium mizutaii* was isolated from the vineyard dump region, which was found to break down organophosphate pesticides *in-vitro* conditions. The degradation efficiency was found by spectral analysis in UV visible spectra through pre and post degradation. Gas chromatography with Mass spectroscopy identified the breakdown products as amine bis 2-phosphoethyl, butyric acid 3-amino 4-methoxy, acetamide and phosphoric acid trimethyl ester. These compounds have never been previously reported. This bacterium provided a suitable bioremediation entity in these yards to overcome the pesticide pollution of soils. The *Sphingobacterium mizutaii* has never been previously reported to be used in the bioremediation of organophosphate pesticides. The application of this bacterium in the agricultural sector can have substantial use in the sustainable agriculture.



## 1. Introduction:

Insecticides containing organophosphates are very hazardous to insects, but not to people or domestic animals [1]. Although certain organophosphate pesticides are clearly neurotoxic, such as triorthocresyl phosphate, nipafox, and trichlorfon compounds, the situation is less apparent for other organophosphate pesticides [2]. Factory employees who make these chemicals, as well as agricultural workers who spray crops with them, are at danger. Organophosphate poisoning is uncommon in the United States, although outbreaks have been documented in various Third World nations [3]. Amongst the insecticides used, Quinalphos, an organothiophosphate insecticide is at the top list to control insects such as flea beetles, thrips, mealy bugs, and leaf hoppers on grapes [4].Viticulture has commercial significance, employing frequent organophosphate as one of the agrochemicals in the management of various pests and diseases [5]. Higher

capital gains for pest damage-free crop is the driving force in rampant application of this pesticide. Moreover, the efficiency of these pesticides has marked their potential in crop yield and quality. This residue of these pesticides is thought to cross the safety guidelines in all matrices and urges a need to degrade it for sustainable development in agriculture [6]. There are records of organophosphates in grapes [7], wine [8] and raisins as well [9]. Most of the farm remnants from vineyard are dumped at a single location and let to decompose. This creates a nitrogen-rich condition for bacteria that certainly grows with the applied organophosphate pesticides. This generates a possible natural hotspot for organophosphate pesticide degrading bacteria.

Organophosphates like Quinolphos degrade comparatively faster with combined action of biotic and abiotic factors. Microorganisms in the environment breakdown most organophosphate insecticides as a supply of their limiting elements, carbon (C) and/or phosphorus (P). Oxidation, hydrolysis, alkylation, and dealkylation are the main processes involved in the degradation process [10]. The adsorption mechanism is aided by abiotic hydrolytic degradation [11]. Photodegradation of various organophosphates is also observed [12]. Though, hydrolysis is a thoroughly studied pathway in organophosphate degradation, particularly caused by cleavage of P-S or P-I bond [13]. Enzymatic hydrolysis specifically is gaining interest in Microbiology due to the manageable mode of application. Organophosphorus acid anhydrases are another name for these enzymes, which have previously been called paraoxonase, esterase, phosphotriesterase, diisopropyl fluorophosphatase, somanase, and parathion hydrolase [14]. The existence of the *oph* gene in the cells of bacteria that degrade organophosphorus chemicals, as well as hydrolase as the primary enzyme involved in the process, has been emphasized by a number of studies [15]. Chlorpyrifos, dichlorvos, diazinon, fenamiphos, fenitrothion, isofenophos, parathion, phorate, malathion, parathion-methyl, monocrotophos, profenophos, and other organophosphate pesticides have all been extensively used, and their environmental fate and bacterial degradation methods have all been extensively studied. Many bacterial and fungal strains have been reported to degrade nearly all of the organophosphate pesticides [16].

After the crop harvest, the pesticide residues are transmitted from the fallen leaves into soil, which turn into nutrients for the bacteria. In the condition of nutrient limitation, these pesticides are taken up by the bacteria as source of C and P, to assimilate them into respiratory cycle and produce energy. Harnessing the bacterial potential to degrade these pesticides can reduce the health risks, recalcitrancy and increase the sustainability of agricultural land. It is essential that the rate of conversion of pesticide into sub molecules be high to render an economic feasibility of application in agriculture. The identification of molecules after enzymatic degradation can provide an idea of biochemical pathway in biodegradation. Quantification of such degraded molecules would also aid in finding the efficacy of the bacteria and can be compared with potential of other recorded bacteria.

## 2. MATERIALS AND METHODS:

### 2.1 Chemicals:

Analytical grade Quinalphos (o, o-diethyl, o-quinoxalin-2-yl-phosphorothioate) and Seubert's mineral salts medium prepared with composition described by Seubert [17] with all the chemicals purchased from Sigma-Aldrich India Pvt Ltd., Bangalore, Karnatak, India.

### 2.2 Organisms and Growth conditions:

Bacterial samples were isolated from soil taken from pesticide-contaminated vineyard dump region of taluka Vita, district Sangli. The enrichment was done on media with quinalphos as the sole carbon source. It was grown on a rotary shaker (150 rev $min^{-1}$ with 500 ml flasks) at room temperature on Seubert's mineral salts medium (MSM) [17] containing 20 mg/L quinalphos as a sole source of carbon. At 600 nm, turbidometry was used to measure growth. Slants of quinalphos–mineral salts agar were used to keep the colonies alive. The colony

characters, cultural, and biochemical properties of the quinalphos-degrading organism were used to determine its identity [18].

**2.3 Identification of Bacterial Isolate By 16S rRNA Gene Sequencing:**

The bacterial 16S rRNA gene was amplified from the total genomic DNA using universal eubacteria specific primers 8F (5'GAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3'), which yielded a product of 1424 base pairs. The settings for the polymerase chain reaction (PCR) were 35 cycles of 95°C denaturation for 1 minute, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute, plus one cycle of 72°C extension for 7 minutes. PEG-NaCl precipitation was used to purify the PCR product (Sambrook et al. 1989). The PCR product was combined with 0.6 volume of PEG-NaCl solution (20% PEG 6000, 25 mol/l NaCl) and incubated at 37°C for 10 minutes. Centrifugation at 12,000 rev/min for 10 minutes was used to collect the precipitate.

The pellet was washed twice in 70% ethanol and vacuum-dried before being resuspended in glass distilled water at a concentration of >01 pmol m/1. A Big Dye terminator kit (Applied Biosystems, Inc., Foster City, CA) was used to sequence the purified product directly [19]. The automated sequencer AB1- PR1SM was used to execute the sequencing reactions (ABI-373xl genetic analyser). The nucleotide sequence analysis was done using the NCBI server's Blastn tool (www.ncbi.nlm.nih.gov/BLAST). The CLUSTALW software VI.82 at the European Bioinformatics site (www.ebi.ac.uk/clustalw) was used to align the sequences. Ribosomal Database Project (RDP) II (http://rdp.cme.msu.edu) was used to analyse the 16S rRNA gene sequence.

To reduce ambiguities, the sequence was manually refined after cross-checking with the original data. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary tree was obtained. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [20]. The phylogenetic tree was drawn to scale, with branch lengths in the same units as the evolutionary distances used to construct the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. This analysis involved nine nucleotide sequences. Pairwise deletion option was selected to remove all ambiguous positions for each sequence pair. Evolutionary analyses were conducted in MEGA X.

**2.4 Utilization of Quinalphos by Isolate:**

By measuring growth in MSM containing 20 mg/L of Quinolphos, the capacity of the isolate WLDQ20 to use Quinolphos as a single carbon source was assessed. The rate of consumption of quinalphos during the development of WLDO20 was evaluated using a UV-visible spectrophotometer to quantify the reduction in UV absorbance at 235 nm. To test whether physical conditions altered the transformation of quinalphos, uninoculated control was used. After 120 hours of incubation, the rate of degradation of quinalphos was evaluated at varied pH (5.0–9.0) and temperature (18–36°C). Triplicates were taken to find the (SD).

**2.5 Identification of metabolite by GC-MS:**

The metabolites were extracted from culture filtrates of the organism cultured on quinalphos with ethyl acetate and analysed on silica gel G plates using thin-layer chromatography (TLC) with ethyl acetate–hexane (4: 1, v/v). The metabolites were detected using ultraviolet (UV) light (at 254 nm), iodine vapours, and spraying with a 1% $FeCl_3$–$K_3Fe(CN)_6$ solution in water. When aromatic compounds were sprayed with diazotized p-nitroaniline or Gibbs reagent, they took on their distinctive colour (2% solution of 2, 6-dichloroquinone-4-chlorimide in methanol). Arnow's reagent was used to identify ortho-dihydroxy chemicals [21]. Spraying the TLC with boric acid – citric acid (05 g each) in 20 ml methanol and heating at 100°C was used to identify 2-hydroxyquinoxaline [21]. The usual approach described in the literature was used to detect nitrite ions in the culture filtrate [22]. Ellman reagent was used to identify thiol compounds [23]. The isolated

spots were then re-extracted analyzed using GC-MS. The GC-MS system has a quadrupole mass filter Rtx-5MS capillary column (30 m x 0.25 mm), a 0.5-second scan interval, and a mass range of 40 to 500. The temperature programme was held at 50°C for 1 minute with 20°C increase for 1 minute to a final temperature 280°C for 14.5 minutes, and the injector temperature was kept at 250°C. The injection volume was 1 µl, and the carrier gas was Helium. The mass spectrometer was set to run at 70 eV electron ionization energy. The components were analyzed and compared with NIST library. Retention time (RT), molecular weight and identified metabolite names were recorded.

**3. Results:**

**3.1 Identification of Isolate by 16S rRNA Gene Sequencing:**

The sequence of the isolate is given in Appendix 1. Amplicon of 1424 bp was obtained after PCR amplification. The sequence had the highest homology with *Sphingobacterium mizutaii* sp. clone DQ20 16S ribosomal RNA, partial sequence, GenBank Accession Number: KT427395.1) with 100 percent query coverage and 0.0 E value. The identified 16S ribosomal sequence was registered in GenBank with the Accession Number: NR_042134.1). The morphological and colony characters of the bacteria were also recorded to be gram-negative, short rod-shaped bacteria with large, yellowish, circular, and shiny raised colonies on Nutrient Agar plates. The characters revealed the genus to be *Sphingobacterium* as well. The results of Megablast are shown in the Appendix 2. The phylogenetic study was also performed (Figure 1).

**3.2 Optimal Degradation of Quinalphos:**

The maximal growth as found by absorbance at 600 was found to be at pH 7.2. The optimum temperature was 27°C and gave the highest growth. The optimal degradation graphs are also reported (figure 2). The best degradation percent was found after 7 days of incubation.

At pH 7 and 27°C, the growth was analyzed and spectral scan showed reduction in the quinolphos content. The absorbance before fermentation gave a higher absorbance at 235 nm. The absorbance maxima after fermentation was also at the same wavelength, but the absorbance was low as compared to one before fermentation. The lower absorbance proved that the Quinolphos concentration showed a gradual reduction in the content. The graphical representation of the absorbance is recorded (figure 3).

**3.3 GC-MS Analysis of Samples:**

The TLC of the media sample was run along with control sample containing Quinolphos. Two distinct visible spots were developed. The sample showing Rf value similar to that of control was discarded. The GC-MS results with 4 metabolites Amine, bis (2-phosphino-ethyl) with RT 6.515 min (molecular weight 137), butyric acid, 3-amino-4-methoxy with RT 7.203 min (molecular weight 194), acetamide, N-(4-hydroxycyclohexyl)-cis with RT 7.338 min (molecular weight 157) and Phosphoric acid, trimethyl ester with RT 13.654 min (molecular weight 140) were obtained.

**3.4 Discussion:**

Based on physical and biological characteristics, as well as phylogenetic analyses based on 16S rRNA gene sequences, the quinalphos-degrading organism was identified as *Sphingobacterium mizutaii*. The strain was capable of digesting a variety of organophosphate insecticides, including quinalphos. The organism's only source of carbon and energy was quinolphos at a concentration of 2 mmol/L. At pH 7.2 and 27°C, the ideal fermentation degraded at 89%.

Only two spots were discovered by TLC, one of which was an undegraded Quinolphos that was deselected. The other spot chosen on chromatogram was a combination of degraded quinolphos that was isolated, purified, and identified by GC-MS. The most probable pathway of degradation should be enzymatic degradation by the bacteria. There are no reports of *Sphingobacterium mizutaii* degrading Quinolphos enzymatically.

Many studies are published on the microbial breakdown of organophosphate insecticides [24-28]. *Enterobacter strain B-14* was found to degrade chlorpyrifos by hydrolysis, yielding 3, 5, 6-trichloro2-

pyridinol and diethylthiophospate, which were used as carbon and energy sources [29]. Although the organophosphate insecticide quinalphos has been widely used, its degradation has received little attention. In the presence of glucose, a *Pseudomonas* strain has been demonstrated to digest larger concentrations of quinalphos; however, its degradative route was not been explored [4]. To our knowledge, this is the first report on the bacteria *Sphingobacterium mizutaii* degrading quinalphos.

Bacterial organophosphate hydrolase has been found to breakdown a broad spectrum of organophosphate chemicals (30-34). Theriot and Grunden [35] reported that microbial breakdown of organophosphate chemicals by hydrolysis to be the most important stage in their detoxification since it reduces their toxicity in mammals' systems [20]. Quinalphos was reported to be degraded by hydrolysis by the isolate *Sphingobacterium mizutaii*, which led to their detoxification. This strain might be effective in the bioremediation of hazardous organophosphate pesticide-contaminated soil.

**4. Conclusion:**

A novel *Sphingobacterium mizutaii* strain DSM 11724 was identified and the 16S rRNA sequence was deposited in GenBank with accession number NR_042134.1. There are no previous reports of the identified bacterium with Quinolphos degradation abilities. The maximal degradation protocols helped find the best pH and temperature for Quinolphos degradation. The concentration of pure quinolphos was found to be reduced substantially. The GC-MS also revealed intermediate degradation metabolites that were never reported previously. The future prospects can be optimization of media for maximal degradation, identifying the pathway of Quinolphos degradation and identifying genes and their expression in presence of Quinolphos as sole energy source.

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**Appendix 1**

**Table 1 Isolated bacterial 16S rRNA gene sequence**

| **Amplicon sequence** |
|---|
| CTAATACATGCAAGTCGGACGGGATCCATCGGTAGCTTGCTACCGATGGTGAGAGTGGCGCACGGGTGCGTAACGCGTGAGCAACC |
| TGCCCATATCAGGGGGATAGCCCGGAGAAATCCGGATTAACACCGCATGACACTGCTTTCCGGCATCGGGAGGTGGTCAAATATTCA |
| TAGGATATGGATGGGCTCGCGTGACATTAGCTAGTTGGTGGGGTAACGGCCCACCAAGGCGACGATGTCTAGGGGCTCTGAGAGG |
| AGAATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTAAGGAATATTGGTCAATGGGGGCAACCCTG |
| AACCAGCCATGCCGCGTGCAGGACGACTGCCCTATGGGTTGTAAACTGCTTTTGTTAGGGAATAAACCCCGCTACGTGTAGCGGGCT |
| GAATGTACCTAAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCGAGCGTTATCCGGATTTATTG |
| GGTTTAAAGGGTGCGTAGGCGGCACTTTAAGTCAGGGGTGAAAGACGGCAGCTCAACTGTCGCAGTGCCCTTGATACTGAAGTGCT |
| TGAATGCGGTTGAAGACGGCGGAATGAGACAAGTAGCGGTGAAATGCATAGATATGTCTCAGAACACCGATTGCGAAGGCAGCTG |
| TCTAAGCCGTTATTGACGCTGATGCACGAAAGCGTGGGGATCGAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGATG |
| ACTCGATGTTTGCGATATACCGTAAGCGTCCAAGCGAAAGCGTTAAGTCATCCACCTGGGGAGTACGCCCGCAAGGGTGAAACTCA |
| AAGGAATTGACGGGGGCCCGCACAAGCGGAGGAGCATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCCGGGCTTGAAAGTT |
| ACTGAAGGGCGCAGAGACGCGCCCGTCCTTCGGGACAGGAAACTAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTG |
| GGTTAAGTCCCGCAACGAGCGCAACCCCTATGTTTAGTTGCCAGCACGTCAAGGTGGGGACTCTAAACAGACTGCCTGCGCAAGCA |
| GAGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGTCCGGGGCTACACACGTGCTACAATGGATGGTACAGCGGGCA |
| GCTACACAGCAATGTGGTGCCAATCTCGAAAAGCCATTCACAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTTGGATTCGC |
| TAGTAATCGCGTATCAGCAATGACGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGCCATGAAAGCTGGGGGTACC |
| TAAAGCATGTAACCGCAAGGAGCGTGTTAGGGTAAAACCGGTAATT |

**Table 2 Megablast analysis of query sequence in NCBI BLAST**

| Description | Scientific Name | Max Score | Total Score | E | % ident | Acc. Length | Accession |
|---|---|---|---|---|---|---|---|
| Uncultured Sphingobacterium sp. clone DQ20 16S ribosomal RNA gene, partial sequence | uncultured Sphingobacterium sp. | 2630 | 2630 | 0 | 100 | 1424 | KT427395.1 |
| Uncultured bacterium clone PE28 16S ribosomal RNA gene, partial sequence | uncultured bacterium | 2630 | 2630 | 0 | 100 | 1479 | AY838484.1 |
| Sphingobacterium mizutaii strain DSM 11724 16S ribosomal RNA, partial sequence | *Sphingobacterium mizutaii* | 2630 | 2630 | 0 | 100 | 1491 | NR_042134.1 |
| Flavobacterium sp. strain Bacter-13 16S ribosomal RNA gene, partial sequence | Flavobacterium sp. | 2625 | 2625 | 0 | 99.93 | 1481 | MH671385.1 |
| Uncultured bacterium gene for 16S rRNA, partial sequence, clone: G3CLN22 | uncultured bacterium | 2625 | 2625 | 0 | 99.93 | 1450 | AB696255.1 |
| Sphingobacterium mizutaii strain LMG 8340 16S ribosomal RNA, partial sequence | *Sphingobacterium mizutaii* | 2625 | 2625 | 0 | 99.93 | 1454 | NR_118144.1 |
| Uncultured bacterium clone PE25 16S ribosomal RNA gene, partial sequence | uncultured bacterium | 2625 | 2625 | 0 | 99.93 | 1494 | AY838481.1 |
| Sphingobacterium mizutaii strain AV5 16S ribosomal RNA gene, partial sequence | *Sphingobacterium mizutaii* | 2623 | 2623 | 0 | 99.86 | 1501 | KX436993.1 |
| Sphingobacterium mizutaii strain NF 296 16S ribosomal RNA gene, partial sequence | *Sphingobacterium mizutaii* | 2623 | 2623 | 0 | 99.86 | 1479 | JF708890.1 |

**Note:** All the accessions had query cover of 100%, column E is E-value of the aligned sequences, % ident is percent identity of the sequence of the test Bacterial amplicon.

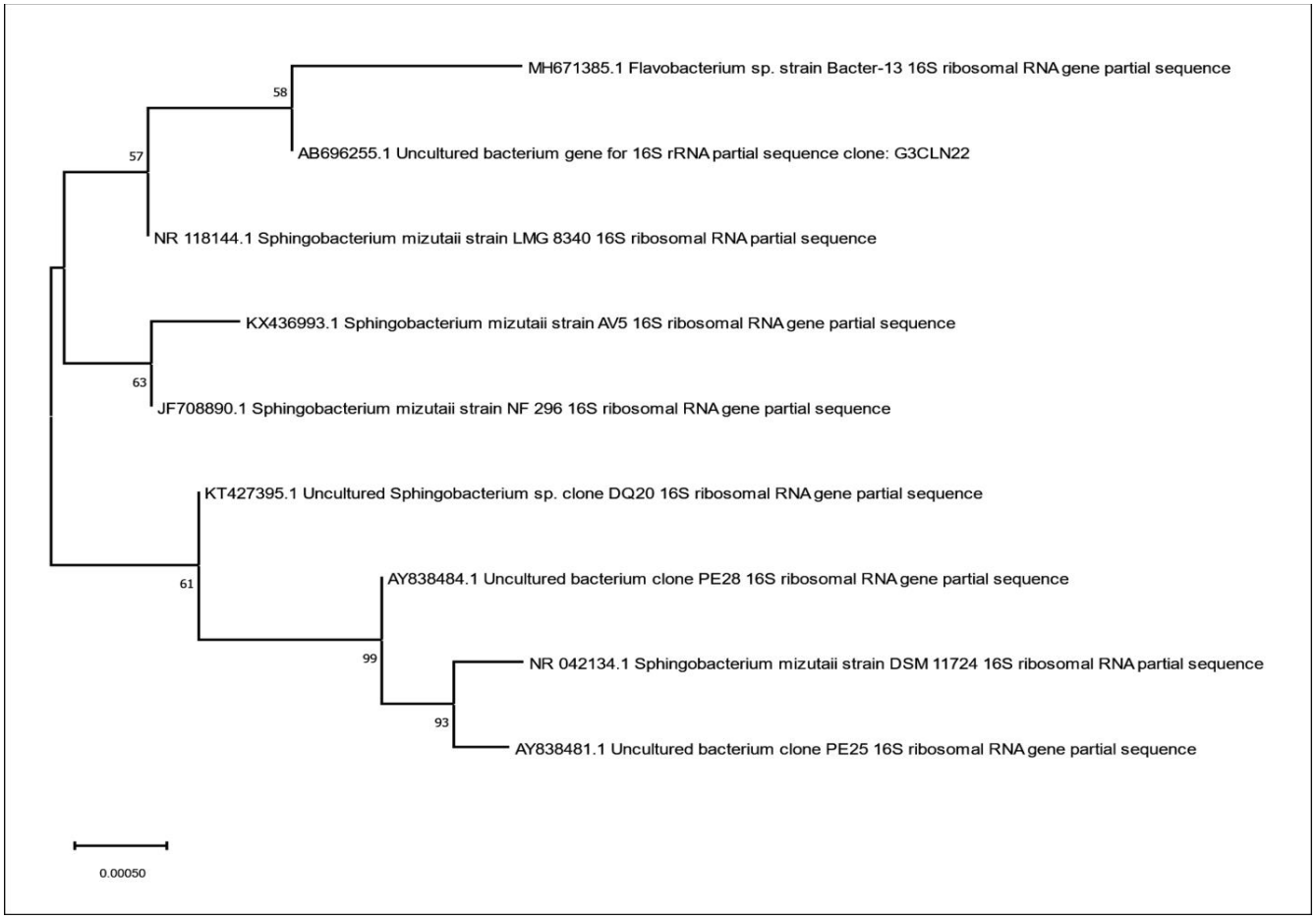


**Figure 1 Phylogenetic position of isolated strain *Sphingobacterium mizutaii DQ20* based on 16S rRNA gene sequence. Distances option according to p-distance method and clustering with neighbour-joining performed using MEGA X [20]. 50% bootstrapping based on 1000 replications are given at branch point. Bar, 0.1 nucleotide substitution per nucleotide position.**

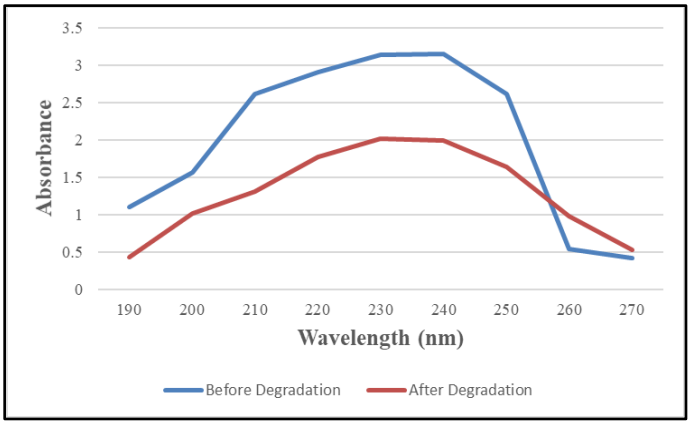


**Figure 2: Effect of $P^H$ and temperature on degradation percentage of Quinolphos.**

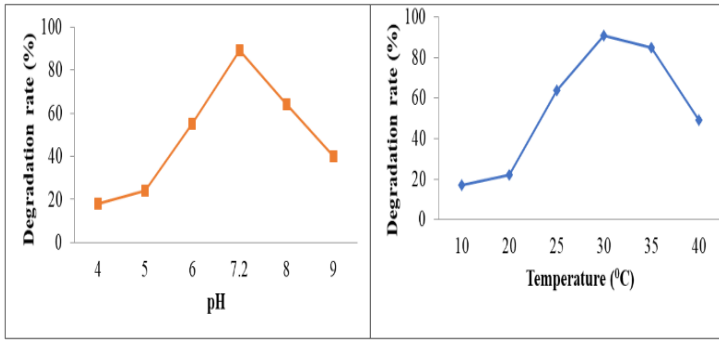


**Figure 3: Spectra of Quinolphos by UV-Vis analysis after fermentation of media.**