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## Effect of Imidacloprid on Bacterial Soil Isolate *Bacillus weihenstephanensis*

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Additional information is available at the end of the chapter

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### Abstract

Imidacloprid is a chloronicotinyl insecticide used widely to control biting and sucking insects. The over accumulation of this pesticide in environment requires higher awareness about this pesticide. Present investigation was carried out to analyze the effect of imidacloprid on antioxidant enzymes such as superoxide dismutase, catalase and peroxidase in soil isolate *Bacillus weihenstephanensis* isolated after laboratory and field studies on the toxic effect of imidacloprid. Further, the genes for the three enzymes involved in the antioxidant defense process in soil isolate *Bacillus weihenstephanensis* were sequenced and identified. Study on the effect of  $10^{-7}$  to  $10^{-3}$  molar concentrations of imidacloprid for a period of 24, 48, 72 and 96 h on three antioxidant enzymes superoxide dismutase, catalase and peroxidase in *Bacillus weihenstephanensis* showed that there was an increase in the activity of all the three antioxidant enzymes. The enzyme activity increased with an increase in the concentration of insecticide proving that the inhibitory effect is dose-dependent. Further, sequencing revealed that Fe/MnSOD (sod A), hydroxyperoxidase HP(II) (Kat E) and glutathione peroxidase genes were expressed in response to stress induced by imidacloprid treatment in *Bacillus weihenstephanensis*. The present investigation indicates that imidacloprid induces the expression of antioxidant enzymes in the soil isolate *Bacillus weihenstephanensis*. The synthesis of antioxidant enzymes may be helping *Bacillus weihenstephanensis* in resisting the toxic effects of imidacloprid.

**Keywords:** Imidacloprid, *Bacillus weihenstephanensis*, Antioxidant enzymes, Genomics

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### 1. Introduction

The insecticides have been used over the past 40 years for protection of crops against insects. Most insecticides cause pollution of air, soil and water due to application by spraying in large

quantities. The use of pesticides has become an integral part of the modern agricultural system. It is estimated that 4 million tons of pesticides have been applied to world crops annually for pest control [1]. The residual pesticides may become the contamination sources and pose a serious threat to the soil and groundwater environment through the rainfall infiltration process. Some pesticides act on biochemical processes that are common to many animals, plants and microorganisms, and thus are a greater hazard to non-target organisms. Imidacloprid is a systemic chloronicotinyl insecticide and is used for soils, seeds and foliar applications for the control of sucking insects, including rice hoppers, aphids, thrips, whiteflies, termites, turf insects, soil insects and some beetles [2]. The active chemical works by interfering with the transmission of stimuli in the insect's nervous system [3]. Imidacloprid is a Category II acute toxicant, and thus, is classified as a General Use Pesticide. Imidacloprid is hazardous to the insects, especially honeybees, and also to fish, birds and algae.

In humans it is linked to reproductive and mutagenic effects and is considered neurotoxic. Reproductive toxicity testing also showed that imidacloprid is an agonist to the acetylcholine receptors that regulates the endocrine system in the brain [4]. The over accumulation of this pesticide in environment requires higher awareness about this pesticide. Imidacloprid is reported to have different impacts on soil bacterial community and also cluster analyzing clearly showed that imidacloprid has significant negative impact on soil bacterial diversity in highly polluted farms and soil microbial balance has been gradually upset by the application of more pesticide.

Oxidative stress is a misbalance between reactive oxygen species (ROS) generation and detoxification, resulting in increased levels of enzyme activity. ROS are of increasing interest in environmental toxicity as they may provide insights to toxicity mechanisms and may identify novel biomarkers. ROS can modify and inactivate proteins in a variety of ways. It is commonly recognized that *Escherichia coli* is the most suitable model system for the investigation of the cell response to oxidative stress. When organisms or cells are exposed to low levels of certain harmful physical or chemical agents, the organisms acquire an induced tolerance against the adverse effects. The effect of hydrogen peroxide on the activity of Sox RS and Oxy R regulon enzymes in different strains of *Escherichia coli* has been studied. Exposure to acetamiprid in *Escherichia coli*, *Pseudomonas* and *Bacillus subtilis* resulted in synthesis of stress enzymes [5].

The term "genomics" was first used by Winkler to describe the haploid set of chromosomes and the genes associated with them. Genomics includes many scientific disciplines [6]. Toxicogenomics is the subdiscipline combining the fields of genomics and toxicology [7]. It has also been described as the study of genes and their products important in adaptive responses to chemical-derived exposures. The toxicogenomic approach provides opportunities to improve understanding of the molecular mechanisms underlying toxic responses to environmental contaminants [8]. Therefore, the present investigation was carried out to study the effect of  $10^{-7}$  to  $10^{-3}$  molar concentrations of imidacloprid for a period of 24, 48, 72 and 96 h on three antioxidant enzymes superoxide dismutase, catalase and peroxidase in *Bacillus*

*weihenstephanensis*. Further, the genes for the three enzymes involved in the antioxidant defense process in soil isolate *Bacillus weihenstephanensis* were sequenced and identified.

## 2. Materials and methods

### 2.1. Laboratory experiment

The experiment was carried out during the summer of 2011 at the laboratory of Department of Biotechnology and Microbiology, Karanatak University, Dharwad, Karnataka. The soil samples were collected from cotton fields around Hubli city. These fields did not have a history of imidacloprid applications for the past 5 years. Soil was collected at a depth of 15 cm and samples were passed through a sieve of 2 mm to remove stones and plant debris. One gram of soil was mixed with 9 ml of sterilized water and mixed by shaking for even distribution of soil in water. And 1 ml of solution from this test tube was then added to another test tube with 9 ml sterilized water. This gives a dilution of  $10^{-2}$  and in the same pattern dilutions up to  $10^{-7}$  were prepared. And 100  $\mu$ l of solution from  $10^{-6}$  dilution was spread on nutrient plates containing different concentration (125, 250, 500 and 1,000 ppm) of imidacloprid. These plates were incubated at 37°C for 48 h. After incubation, bacterial colonies were counted using colony counter and results were expressed as the number of bacteria in 1 g of soil [9].

### 2.2. Field experiment

Imidacloprid was applied to experimental field at recommended rates and at 1.5 $\times$  rates on two plots on same field in replicates, the plot without application served as control. Soil samples were taken on 7, 14, 21 and 28th day of application. About 1 g of sample was suspended in 9 ml of sterilized water. Serial dilutions were done as mentioned earlier. Then, 100  $\mu$ l of solution from  $10^{-6}$  dilution was spread-plated on nutrient agar plates. These plates were incubated at 37°C for 48 h. After incubation, colonies of bacteria were counted using colony counter and results were expressed as the number of bacteria in per gram of soil [9].

### 2.3. Preparation of stock solution of imidacloprid

The stock solution of one molar imidacloprid was prepared and further diluted to give  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  molar. Soil isolate was isolated and identified from soil as described in our previous publication. The bacterium was maintained at 4°C on nutrient agar and sub-cultured every fortnight. The medium used for toxicity testing was an optimized medium (dextrose – 0.65 g /l; yeast extract – 1.05 g /l; K HPO –0.30 g/l; NaCl – 0.25 g /l) [9].

### 2.4. Preparation of inoculum

Pre-inoculum was prepared by inoculating a loop full of bacteria from the overnight incubated nutrient agar slant cultures on a 100 ml sterilized optimized growth medium and incubated for 24 h at 37°C under static conditions [9].

2.5. Identification of bacterial isolate

Imidacloprid tolerant colonies isolated and identified morphological, cultural and biochemical characters and 16S rDNA identification as described in our previous publication. The pure culture was grown on nutrient agar medium [10].

2.6. Experimental procedures

Five millilitres of the pre-inoculum was inoculated to 250 ml Erlenmeyer’s flask containing 100 ml of sterilized optimized growth medium amended with different molar concentrations of imidacloprid. The flasks were incubated at 37°C for 96 h under shaking conditions at 120 rpm on a rotary shaker. At regular intervals, sample was taken out from each flask aseptically for analysis [10].

2.7. Extraction of enzymes

The cells were centrifuged at 8,000 rpm for 3 min and the pellet was dissolved in 0.2 ml of lysis buffer (50 mM tris-cl and 10 mM lysozyme). The tubes were incubated at 37°C for 10 min and centrifuged at 10,000 rpm for 10 min. Supernatant was used as the source of enzyme.

2.8. Estimation of stress enzyme activity

The activity of SOD, catalase and peroxidase were assayed using the supernatant from centrifugation (15,000 rpm) for 12 min at 4°C homogenate by standard methods [11–12].

2.9. Isolation of genomic DNA

The genomic DNA was isolated from soil isolate SP-03 by CTAB method. The DNA stock samples were quantified using Nanodrop spectrophotometer at 260 and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml. Quality and purity of DNA were checked by agarose gel electrophoresis. The DNA was used further for PCR. And 250 µl of the isolated genomic DNA was taken and treated with 1 µl of RNase enzyme and incubated at 37°C in a water bath for 30 min and further incubated at 60°C for 10 min in the water bath and used as a template with PCR mix [13].

Contents	Volume (µl)
10× Taq Assay buffer	1.0
MgCl <sub>2</sub>	0.5
Template	2.0
Primers (Forward + Reverse) (10 pM)	1 + 1
dNTP mix	0.4
Taq DNA polymerase	0.3
HPLC grade water	3.8

Table 1. Composition of PCR mix

SL. No.	Antioxidant enzyme	Primer
1	Superoxide dismutase (SOD)	Forward- 5-atagcttggcagagcgacat Reverse- 5-tatacgctcattgcagcag
2	Catalase (CAT)	Forward -5-ggaaaccactggcaggtaaa Reverse- 5-ctgccgatctcacttcatca
3	Peroxidase (POX)	Forward- 5-tcacaaccgttcatttcca Reverse- 5-ccagagctgcttcgtaatcc

**Table 2.** Primers of stress enzymes

Steps	Temperature (°C)	Time	Cycles
Initial denaturation	95	2 min	1
Final denaturation	94	30 sec	30
Annealing*		30 sec	
Extension	72	90 sec	
Final extension	72	10 min	1

\*mentioned in text

**Table 3.** PCR Conditions

The annealing temperatures for catalase peroxidase and for superoxide dismutase were 46°C.

## 2.10. Statistical analysis

Statistic significance between the control and the experimental data were subjected to analysis of variance (ANOVA) followed by post-hoc Dunnett's test ( $P \leq 0.05$ ) [9].

## 3. Results

### 3.1. Effect of imidacloprid on soil bacterial populations in laboratory studies

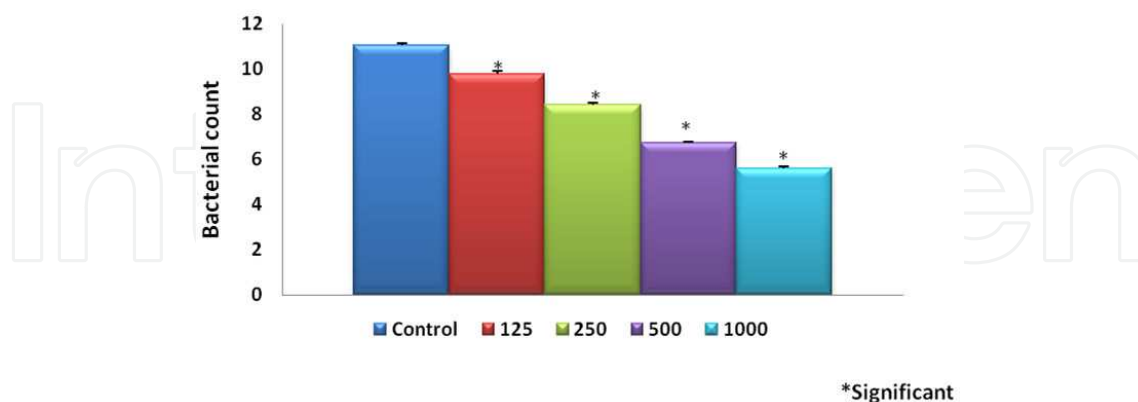
The result of the laboratory study is given in Table 1. Application of imidacloprid at 125, 250, 500 and 1,000 ppm resulted in 9.80, 08.40, 6.73 and  $5.60 \times 10^{-6}$  colonies ( $P < 0.05$ ) when compared to  $11.05 \times 10^{-6}$  in control plates (Graph 1).

### 3.2. Effect of imidacloprid on soil bacterial populations in field studies

In the field studies, imidacloprid was applied in both recommended and  $\times 1.5$  rates and the results are given in Graph 2. The results showed that imidacloprid-treated fields at recommended and  $\times 1.5$  rates showed significant ( $P < 0.05$ ) decline in bacterial counts at different



post-application intervals when compared to control, but the colony count increased with the time.



**Graph 1.** Effect of imidacloprid on bacterial populations in the soil under laboratory conditions

### 3.3. Identification of soil isolate

The bacterial strain SP-03 isolated from soil was a rod-shaped, Gram-positive bacterium, facultatively anaerobic, grows at 5–40°C, at pH 6–7; produce subterminal ellipsoidal endospores; white-colored colonies; positive for catalase activity, Voges-Proskauer, starch hydrolysis and oxidase; and negative for methyl red, gelatin liquefaction, production of indole and citrate. The 16S rDNA gene of SP-03 was isolated and sequenced. This 16S rDNA gene sequence was then compared with previously published 16S rDNA gene sequences and based on matches the strain was classified as a member of the genus *Bacillus*. The sequence of strain SP-03 displayed the highest identity (100%) with the 16S rDNA gene of *Bacillus weihenstephanensis* KBAB4 (GenBank Accession Number: HG 486214.1) (Figure 1, 2). The *Bacillus weihenstephanensis* showed highest growth at 22°C and at pH of 7.0.

### 3.4. Effect of imidacloprid on antioxidant enzymes in *Bacillus weihenstephanensis*

On exposure of *Bacillus weihenstephanensis* to various molar concentrations ( $10^{-3}$  to  $10^{-7}$ ) of imidacloprid for 24, 48, 72 and 96 h, there was a significant ( $P \leq 0.05$ ) increase in the activity of antioxidant enzymes studied. There was a significant increase ( $P \leq 0.05$ ) in the activity of superoxide dismutase (Graph 3), catalase (Graph 4) and peroxidase (Graph 5) in all the treated groups. The antioxidant enzyme activity increased with an increase in the concentration of imidacloprid.

### 3.5. Gene isolation and sequencing of the stress enzymes of *Bacillus weihenstephanensis* on exposure to imidacloprid

In the present gene sequencing study, different markers of 400, 600, 1,000 and 1,200 bp (Figure 6) were run along with our test sample. The superoxide dismutase gene was corresponding to 624 bp. This gene was isolated, eluted and sent for sequencing. The sequence was received and

this was subjected to NCBI BLAST which revealed that the nucleotide sequence was of 624 letters bearing accession number YP\_001648011.1 that showed 100% query coverage with *Bacillus weihenstephanensis* Fe/MnSOD (sod A) (Figure 3). The gene for catalase corresponded band at 800 bp. The isolated gene was eluted and sent for sequencing. The received sequence, subjected to NCBI BLAST revealed that the nucleotide sequence was of 800 letters bearing accession number YP\_001647388.1 and showed 100% query coverage with *Bacillus weihenstephanensis* Hydroxyperoxidase HP(II) (*Kat E*) (Figure 4). The gene for peroxidase that corresponded to 480 bp was isolated, eluted and sent for sequence analysis (Table 2, 3). The sequence report was received, which was subjected to NCBI BLAST which revealed the nucleotide sequence of 480 letters bearing accession number YP\_001644822.1 and showed 100% query coverage with glutathione peroxidase of *Bacillus weihenstephanensis* (Figure 5).

## 4. Discussion

### 4.1. Effect of imidacloprid on soil bacterial populations in laboratory and field studies

Results obtained in laboratory studies showed significant ( $P < 0.05$ ) decrease in bacterial count when compared to that of control. A gradual decrease in bacterial count is observed with increase in concentration of imidacloprid, with minimal count reported at 1,000 ppm. The results obtained were similar to results reported earlier in a study involving five other pesticides [14].

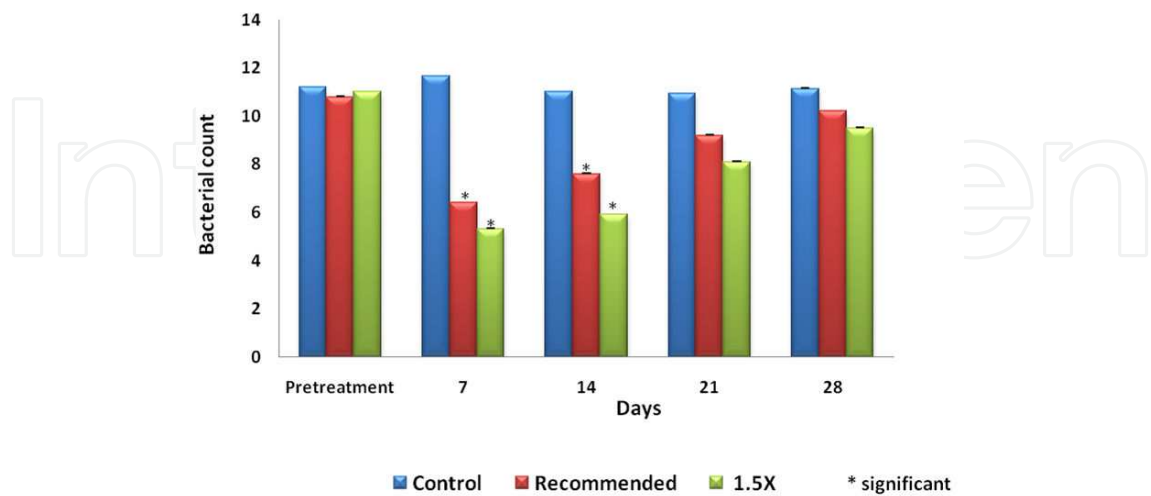
The results indicate toxic effect of imidacloprid on bacterial populations. Results obtained from bacterial enumeration of imidacloprid-treated soils at recommended rate showed significant ( $P < 0.05$ ) decrease in bacterial numbers, proving negative effect of imidacloprid on bacteria. This negative effect reduced after 14 days of treatment. The negative effect of imidacloprid was vanished by 28th day of application, indicated by bacterial count, which was almost similar to pre-treatment count. Similar results were reported in a study involving imidacloprid and five other pesticides; in the study the toxic effect was vanished by 21st day of imidacloprid application [14].

The different studies have shown that the impact of pesticides application microorganisms present in soil is variable. The impact depends on interaction between microorganisms and active substances and formulation. It also depends on surfacing of specific group of microorganisms [15]. The microorganisms can develop the ability to use an applied pesticide as a source of energy and growth [16].

The initial decrease in bacterial count is expected as pesticides are known to affect the microbial populations by controlling the survival and reproduction of individual species. Initial reduction in microbial count is also reported in studies involving different pesticides such as endosulphan, cypermethrin, thiodan, etc. [12, 17–18], and herbicides like glyphosate, atrazine, simazin and alachlor [19–22] when applied at recommended rates. It has been observed in many studies that pesticides stimulated the mineralization rate of organic carbon in comparison with control samples [23–24]. Microorganisms susceptible to toxic effects of pesticides are



removed from the population of soil microflora. The pesticides kill the bacterial cells by penetration and disturbing the cell metabolism.



**Graph 2.** Effect of imidacloprid on bacterial populations in soil under field conditions

The reduction in the number of sensitive microorganisms and increase in resistant microorganisms lead to reduced soil microbial biodiversity. The increase in bacterial numbers after 14th day may be due to the ability of bacteria to degrade toxic compounds like pesticides [25]. The growth of pesticide-resistant microorganisms may compensate the loss of pesticide-sensitive microorganisms in the population [24]. The addition of fungicide leads to increase in bacterial populations due to no competition with fungi or antagonistic inhibition by fungi [26]. Bacteria are known to become resistant to toxic compound with production of specific degrading enzymes [27].

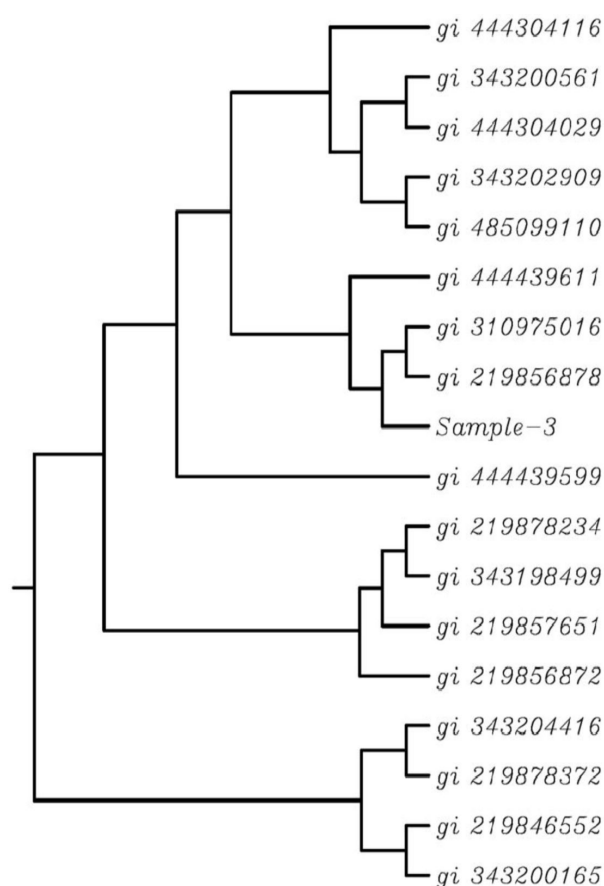
The application of 1.5× of imidacloprid showed significant ( $P < 0.05$ ) decrease in bacterial number. The results were similar to recommended rates, but bacterial numbers increased slowly. These results were comparable to the results reported in similar studies with pesticides like metoalchlor, atrazine, dimethoate and endosulfan [20, 22, 28–29].



**Figure 1.** *Bacillus weihenstephanensis*

## 4.2. Identification of soil isolate

The bacterial strain SP-03 isolated from soil was a rod-shaped, Gram-positive bacterium, facultatively anaerobic, grows at 5–40°C, at pH 6–7; produce subterminal ellipsoidal endospores; white-colored colonies; positive for catalase activity, Voges-Proskauer, starch hydrolysis and oxidase; and negative for methyl red, gelatin liquefaction, production of indole and citrate. The 16S rDNA gene of SP-03 was isolated and sequenced. This 16S rDNA gene sequence was then compared with previously published 16S rDNA gene sequences and based on matches the strain was classified as a member of the genus *Bacillus*. The sequence of strain SP-03 displayed the highest identity (100%) with the 16S rDNA gene of *Bacillus weihenstephanensis* KBAB4 (GenBank Accession Number: HG 486214.1). The *Bacillus weihenstephanensis* showed highest growth at 22°C and at pH of 7.0.



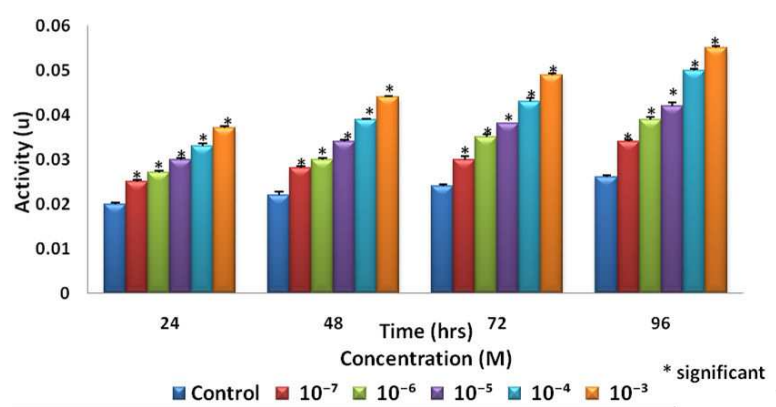
**Figure 2.** Phylogenetic tree of *Bacillus weihenstephanensis*

## 4.3. Antioxidant enzymes

Partial reduction of oxygen to water during microbial respiration gives rise to reactive oxygen intermediates, e.g. superoxide radicals, hydrogen peroxide and hydroxyl radicals. Microorganisms have developed efficient enzymatic and nonenzymatic mechanisms to eliminate these toxic and mutagenic reactive oxygen species. Superoxide is eliminated by dismutation to

H<sub>2</sub>O<sub>2</sub> catalyzed by superoxide dismutase and accumulation of H<sub>2</sub>O<sub>2</sub> is prevented by the action of catalases and peroxidases [30].

Numerous pesticides such as paraquat, DDT, PCB, Arochlor, etc. have been used as model factors inducing oxidative stress both in vivo and in vitro [31]. The tissue damage occurs due to conversion of pesticides to free radicals or superoxide radical during their metabolism. Organisms exposed to different concentrations of xenobiotics have the risk of carcinogenic effect, neurological actions and brain damage [32]. The organisms have developed some mechanisms to control the amount of hydroxyl and superoxide radicals generated to overcome the toxic effects of xenobiotics. Antioxidants quickly scavenge the hydroxyl and superoxide radicals generated. The antioxidants can be enzymatic or nonenzymatic which safely interact with free radicals and terminate the chain reactions before vital molecules are damaged. The antioxidant enzymes include catalase, superoxide dismutase (SOD), glutathione reductase, glutathion-S-transferase and glutathione peroxidase [33].



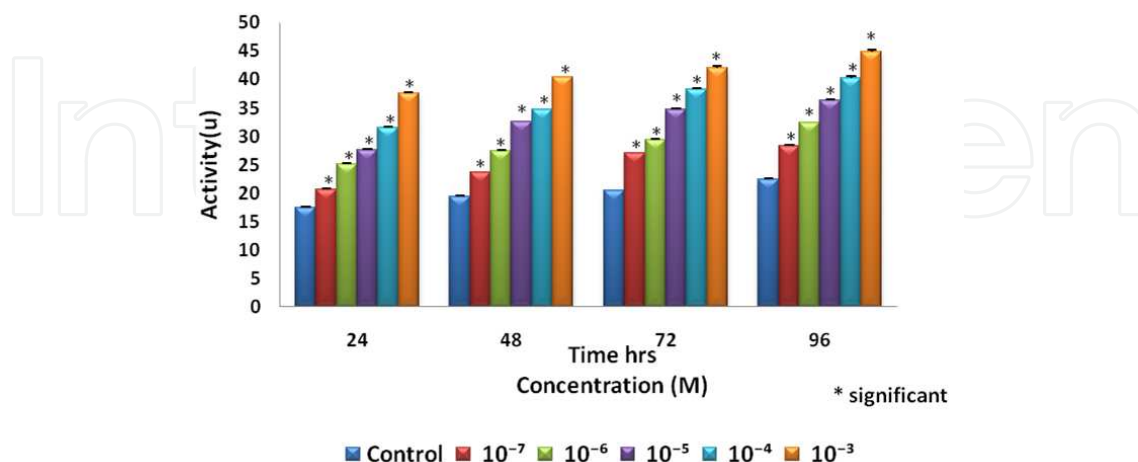
Graph 3. Effect of imidacloprid on SOD activity in *Bacillus weihenstephanensis*

Significant increase in activity of SOD compared with the control may be due to the toxic effects of imidacloprid. ROS depends on the oxidative metabolism of xenobiotics or endogenous compounds. The antioxidant defense systems work by lowering the concentrations of xenobiotics rather than complete elimination. When the ROS generated exceeds the antioxidants' capability of that cell, it results in oxidative stress [34].

The stress-mediated cytotoxicity results due to oxidative processes and loss of key antioxidant enzymes. *Escherichia coli*, *Salmonella typhimurium* and mammalian cells induce antioxidant proteins in response to oxidative stress [35]. It is suggested that an increase in SOD and CAT might be in response to increased oxidative stress or might be due to compensatory response to oxidative stress induced by this xenobiotic. Superoxide dismutase, catalase and peroxidase are the enzymes that participate in the protection against reactive oxygen species.

Catalase is one of the most efficient antioxidants known so far. It is present in peroxisomes of nearly all aerobic cells and protects the cells from the toxic hydrogen peroxide effects by catalyzing its decomposition into molecular oxygen and water without the production of free radicals. In addition, catalase is known to act on toxic compounds by per oxidative reactions.

It is demonstrated that acetamiprid-induced oxidative stress on *Escherichia coli*, *Pseudomonas* sp and *Bacillus subtilis* resulted in elevated superoxide dismutase and catalase activities to antagonize oxidative stress [5].



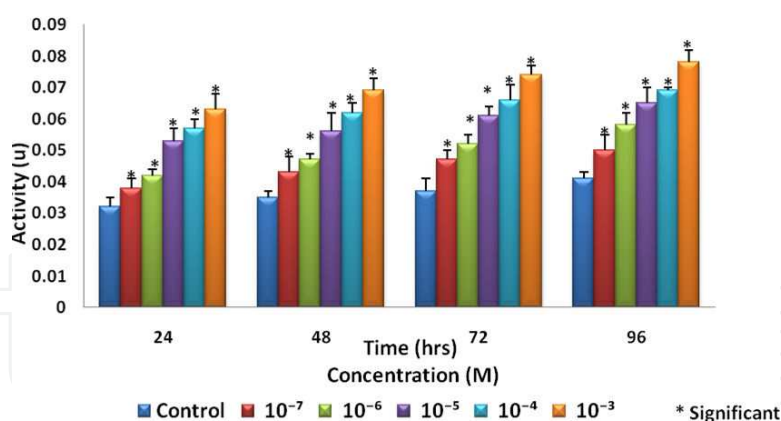
**Graph 4.** Effect of imidacloprid on catalase activity in *Bacillus weihenstephanensis*

The present study revealed that the catalase activity was significantly increased in all the groups with increase in the dose and durational exposure of imidacloprid to *Bacillus weihenstephanensis*. Similarly, it has been reported that induction of major antioxidant enzymes, such as superoxide dismutase and catalase, were observed after their exposure to a single oxygen generating system in *Escherichia coli*. It is suggested that response to low concentrations of hydrogen peroxide induces catalase in *Escherichia coli* during logarithmic growth [36].

Peroxidase is found among animals, plants and microorganisms, where they perform essential roles in the metabolism. To prevent the lethal effects of such metal-ion-catalyzed oxidation (MCO), bacterial cells have evolved protective mechanisms to neutralize the formation of toxic oxygen radicals. For instance, small molecule antioxidants, such as catalases and peroxidases, have been reported to play protective roles in the enteric bacteria in *Pseudomonas* sp. and in *Bacteroides* sp. [37].

The enzyme peroxidase is an important antioxidant enzyme, which plays a pivotal role in plant growth and development. The presence of phenol substances leads to enhanced activity of peroxidase (POD). The POD helps in providing resistance to stress and self-defense by increasing the rate of respiration under stress conditions [38].

The present study revealed that the peroxidase activity in the treated groups increased significantly in higher dose (10<sup>-5</sup>, 10<sup>-4</sup> and 10<sup>-3</sup> M) of exposure and there was no significant increase observed in the lower dose (10<sup>-7</sup> and 10<sup>-6</sup> M) of imidacloprid in *Bacillus weihenstephanensis*. Similar results were reported in other organisms which suggest that a gradual increase of catalase or peroxidase production in aging cultures is not surprising since catalase and/or CP is one of the radical-scavenging enzymes in cells in response to oxidative stress [39]. On the other hand, several organisms produce two or more catalase peroxidase, whereby one



**Graph 5.** Effect of imidacloprid on peroxidase activity in *Bacillus weihenstephanensis*

enzyme was expressed at the end of exponential growth and during the stationary phase. This behavior was observed in *Escherichia coli*, *Pseudomonas putida*, *Streptomyces coelicolor* and *Arcobacter nitrofigilis* [40]. It is also reported that superoxide dismutase and peroxidase form the first line of defense against reactive oxygen species [41].

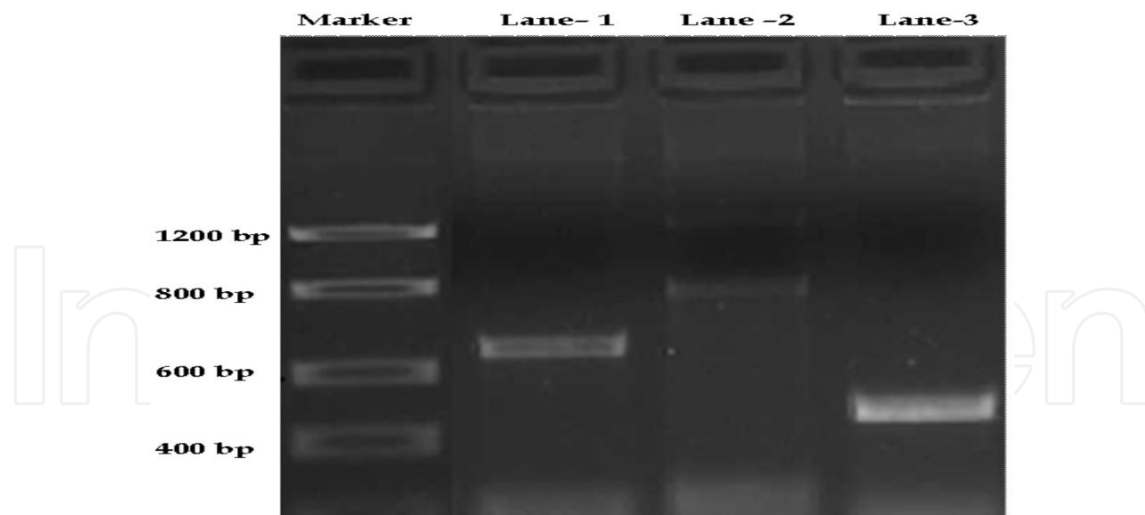
The significant increase in the antioxidant enzymes activity observed in the present study may be due to synthesis of these enzymes as a response to chemical stress induced by imidacloprid or due to inhibition of the membrane-bound enzymes by affecting the enzyme complex, oxidative stress-mediated cytotoxicity enzymes, induction of antioxidant proteins in response to oxidative stress [42].

#### 4.4. Gene sequencing of stress enzymes of *Bacillus weihenstephanensis* on exposure to imidacloprid

The research in life sciences is affected significantly by the mapping of the genes and genomes of organisms. The related mapping technology is changing the current understanding of biological systems [43]. The application of life science areas of toxicology, genetics, molecular biology and environmental health to describe the response of organisms to environmental stimuli is called toxicogenomics. The toxicogenomics is developed in the past 15 years and will help in advancing the scientific basis of risk assessments for the environmental contaminants [44].

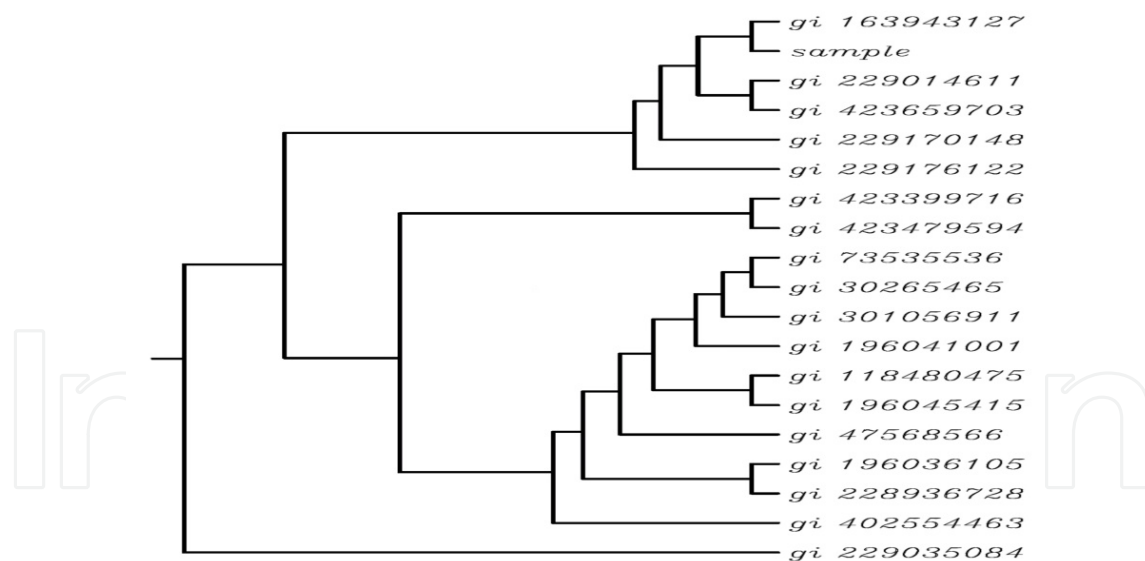
In the present study, the exposure of *Bacillus weihenstephanensis* to imidacloprid resulted in the expression of manganese containing superoxide dismutase (*sod A*) gene. MnSOD and FeSOD have an extremely broad phylogenetic distribution, being expressed in both prokaryotic (eubacterial and archaeal) and eukaryotic cells and are quite homologous [45]. Expression of *sod A* gene has also been reported for other bacterial species. A strain of *Sulfolobus sulfataricus* produced Fe-Mn SOD with half-life of 2 h at 100°C [46]. In a study, superoxide dismutase producing *Bacillus* sp. was isolated from Bulgarian thermal spring [47]. In another study, *Thiobacillus denitrificans* strain "RT" Fe-superoxide dismutase has been purified with a molecular weight of 43,000, and is composed of two identical subunits. Aerobically and anaerobically grown *Thiobacillus denitrificans* cells contain the same Fe-enzyme with similar





**Figure 3.** Gel image of stress enzymes amplicon

levels of activity. Manometric sulfite oxidation measurements suggest for the enzyme a protective function of sulfite against the auto-oxidation initiated by superoxide free radicals [48]. *Escherichia coli* when grown under anaerobic conditions contained only Fe-SOD, but exposure to oxygen induced the synthesis of Mn-SOD and New-SOD [49].



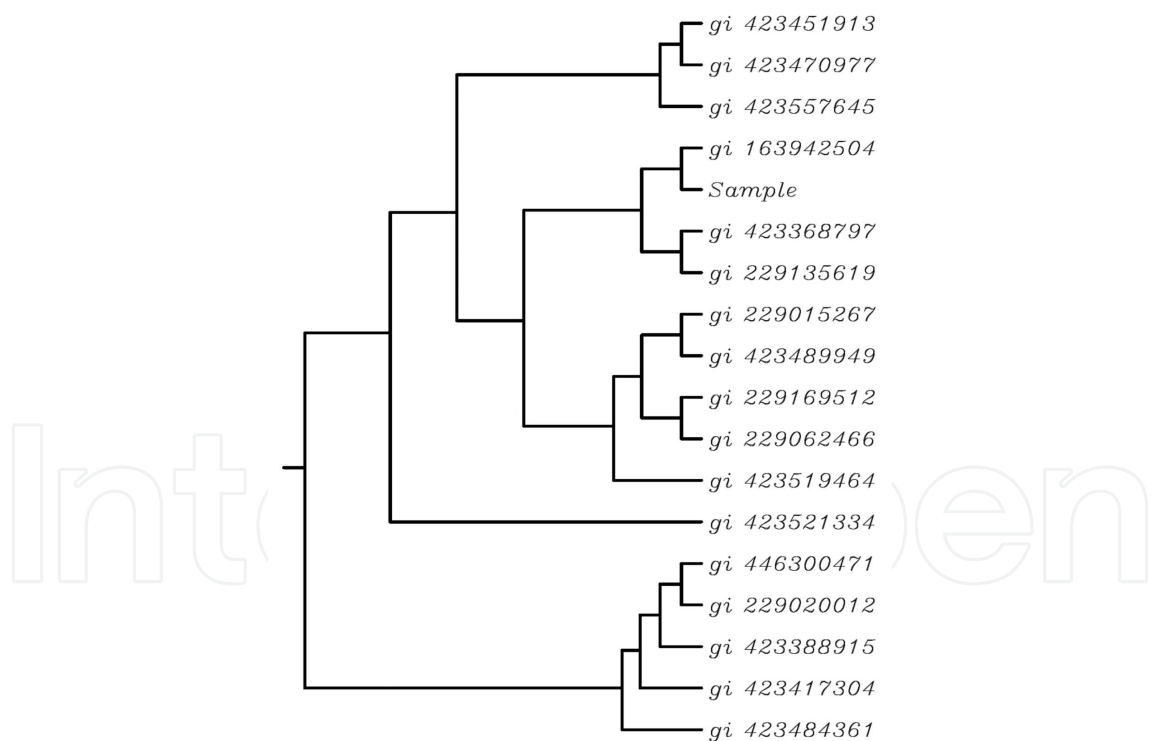
**Figure 4.** Phylogenetic tree of SOD

SOD of *B. subtilis* is manganese associated as indicated by a high similarity of the putative amino acid sequence of *B. subtilis* SodA to those of Mn-Sod from *B. caldotenax* and *B. stearothermophilus*, and presence of four conserved metal-binding sites. This SOD was found in vegetative cells and in spores [50]. A new, thermostable superoxide dismutase (SOD) from *Bacillus licheniformis* M20, is isolated from Bulgarian mineral springs. It is reported that *B.*



*subtilis* contains a cytosolic Mn-superoxide dismutase [51]. Xenobiotic degrading bacteria experience oxidative stress, both as directly from the pollutants themselves and from intermediates generated during biodegradation processes [52]. Depending on the type of oxidative stress, not only different amounts of proteins can be modified but also different species may appear. It was shown that the set of oxidized proteins depended on the method of induction of oxidative stress.

The present study reveals that the genome of catalase encoded in our *Bacillus weihenstephansensis* culture on exposure to imidacloprid was *Kat E* (HPII). HPII and catalase-2 monofunctional catalases of *E. coli* and *B. subtilis*, expressed in the stationary phase, have D-isomer prosthetic groups with six haem. Catalase from *E. coli* HPI R., *Halobacterizlm halobitlm* and facultative alkalophilic *Bacillus* species have bifunctional catalase-peroxidases [53–56]. Several organisms produce two or more catalase/peroxidases, whereby one enzyme was expressed at the end of exponential growth and during the stationary phase. This behavior was observed in *Escherichia coli*, *Pseudomonas putida*, *Streptomyces coelicolor* and *Arcobacter nitrofigilis* [40]. It has been reported that various bacteria such as *Citrobacter freundii*, *Edwardsiella tarda*, *Enterobacter aerogenes*, *Klebsiella pneumonia* and *Salmonella typhimurium* exhibited patterns of catalase activity similar to that of HPI and HPII bands of *Escherichia coli*.



**Figure 5.** Phylogenetic tree of catalase

Bacterial monofunctional catalases of *E. coli* HPII [53] and *B. subtilis* catalase-2, both of which are expressed in the stationary phase, contain six haem D-isomer prosthetic groups in a hexameric structure of larger subunits. From the present study of exposure of *Bacillus weihen-*

*stephanensis* to imidacloprid, it can be concluded that the catalase enzyme can be encoded by the *Kat E* (HP11) gene.

The present sequence analysis of the peroxidase gene suggests that *Bacillus weihenstephanensis* subjected to imidacloprid expressed the glutathione peroxidase (Tpx) gene. The findings of study also support a thiol-dependent antioxidant activity for thiol peroxidase in *Streptococcus parasanguis*, which protects the organism from stress [57]. It is reported that the bacterial thiol peroxidases include a pair of cysteine residues and comprise part of the functional group for the peroxidase activity [58]. *Escherichia coli* thiol peroxidase is part of an oxidative stress defense system that uses reducing equivalents from thioredoxin (Trx1) and thioredoxin reductase to reduce alkyl hydroperoxides [59]. The specific mechanism(s) by which thiol peroxidase protects *Streptococcus parasanguis* from the toxicant may be similar to those described for other thiol-specific antioxidants of *Escherichia coli*. The *Mycobacterium* sp. strain PYR-1 degrades polycyclic aromatic hydrocarbons, environmental pollutants. It was shown that inducible catalase-peroxidase of *katG* gene of this culture is involved in molecular mechanisms of degradation of these pollutants [60].

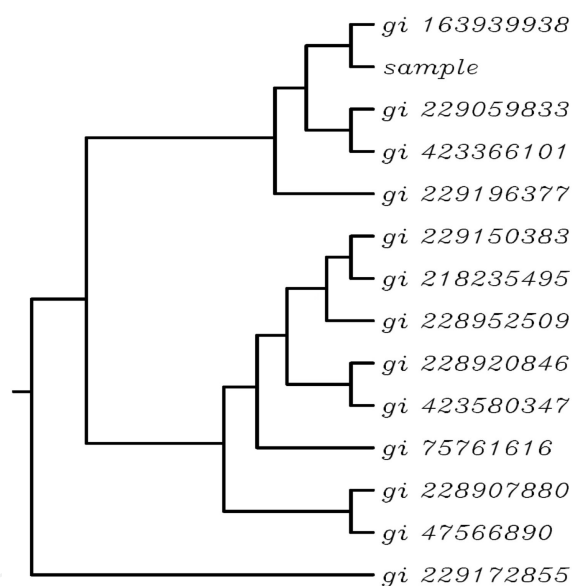


Figure 6. Phylogenetic tree of glutathione peroxidase

## 5. Conclusion

Present investigation was carried out to analyze the effect of imidacloprid on antioxidant enzymes superoxide dismutase, catalase and peroxidase in soil isolate *Bacillus weihenstephanensis*, isolated after field studies on the effect of imidacloprid at recommended and 1.5× rates, Which showed that there was an increase in the activity of all the three antioxidant enzymes. The enzyme activity increased with an increase in the concentration of insecticide proving that the inhibitory effect is dose-dependent. Further, sequencing revealed that Fe/MnSOD (sod A),

hydroxyperoxidase HP(II) (Kat E) and glutathione peroxidase genes were expressed in response to stress induced by imidacloprid treatment in *Bacillus weihenstephanensis*. The present investigation indicates that imidacloprid induces stress, which results in the expression of antioxidant enzymes in the soil isolate *Bacillus weihenstephanensis* to protect the cellular components from oxidative damage. Study also reveals that the soil isolate *Bacillus weihenstephanensis* has developed the resistance to imidacloprid toxicity by synthesis of antioxidant enzymes. Further research can be performed to use it in the field for pollution monitoring and risk assessment due to imidacloprid contamination in soil, thereby exploring the possibility of using soil isolate imidacloprid-resistant *Bacillus weihenstephanensis* in the study of complex biological processes and to clean the fields with imidacloprid contamination.

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