British Biotechnology Journal
4(9): 957-969, 2014

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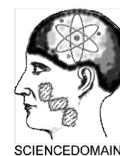

Imidacloprid Induced Intoxication and its Biodegradation by Soil Isolate *Bacillus weihenstephanensis*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AAS designed the study, wrote the protocol and wrote the first draft of the manuscript. Author RBK performed the statistical analysis. Author BBK managed the analyses of the study. All authors read and approved the final manuscript.

Original Research Article

Received 7th December 2013
Accepted 9th March 2014
Published 14th August 2014

ABSTRACT

Aims: Study was carried out to investigate the effect of imidacloprid on biochemical parameters and growth of soil isolate. The imidacloprid degradation by the soil isolate was also studied.
Study Design: The soil isolate was identified and used for toxicity testing. The isolate of Bacillus weihenstephanensis was further tested for its ability to degrade imidacloprid in minimal salt medium (MSM) and tryptic soya medium (TSB). The role of plasmid in imidacloprid degradation was established by curing experiments.
Place and Duration of Study: Department of Biotechnology and Microbiology, Karnatak University, Dharwad, India between June 2011 and December 2012.
Methodology: The soil isolate was identified by morphological, biochemical characters and 16s rDNA identification. Effect of imidacloprid on DNA, RNA, protein, glucose and growth in soil isolate was studied with 10^{-3} to 10^{-7} molar imidacloprid for 96 h. Imidacloprid degradation was determined in MSM and TSB for 28 days with samples taken on 7, 14, 21 and 28th day. The insecticide concentration was tested by HPLC. Plasmid curing was performed.

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Results: The soil isolate was identified as *Bacillus weihenstephanensis*. The study involving soil isolate *Bacillus weihenstephanensis* with 10^{-3} to 10^{-7} molar imidacloprid showed significant (P<0.05) decrease in content of DNA, RNA, protein, glucose and growth. *Bacillus weihenstephanensis* in MSM and TSB showed 46 and 78 % imidacloprid degradation in four weeks. The plasmid of *Bacillus weihenstephanensis* was cured in fourth generation. 18.80% and 75% degradation observed in cured and non cure cells of *Bacillus weihenstephanensis* in TSB.

Conclusion: Study showed that imidacloprid affects the biochemical contents and intern growth of soil isolate *Bacillus weihenstephanensis*. Study also revealed that *Bacillus weihenstephanensis* was able to degrade imidacloprid in MSM and TSB. Further plasmid curing revealed that the genes for imidacloprid degradation are located both in plasmid and chromosome.

Keywords: Imidacloprid; Bacillus weihenstephanensis; growth; biodegradation.

1. INTRODUCTION

Various insecticides to protect crops against insects have been used over the last 40 years. Most insecticides were applied by spraying in large quantities, thus inducing pollution of air, soils and waters. The use of pesticides has become an integral part of the modern agricultural system. It is estimated that 4 million tons of pesticides are 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. It has been estimated that, often, less than 0.1% of pesticides applied to crops actually reaches the target organisms [2].

Toxicity testing of environmental pollutants is being shifted towards the microbial models from animal models in recent years. The microbial models used extensively for toxicity testing are protozoa, algae, fungi and bacteria. Toxicity testing for microbial sensitivity to different environmental pollutants and many microbial systems are being standardized as the indicators of pollutants [3]. The development and standardization of toxicity tests based on prokaryotic (bacteria) or eukaryotic (protozoa, unicellular algae, yeasts) microorganisms instead of higher organisms has enabled fast and inexpensive screening of environmental samples for toxic and genotoxic effects [4].

Xenobitics in soil are treated by bioremediation using microorganisms. This method is used successfully in many countries [5-6]. It used mainly because of its eco-friendliness and cost effectiveness compared to physical and chemical remediation methods [7]. Bacterial species like *Pseudomonas, Flavobacterium, Arthrobacter, Xanthobacter* etc. have been isolated from soil for their capability to degrade pesticides and are used in bioremediation of different pesticides [8]. The soil microorganisms are capable of degrading a wide variety of chemical compounds, from polysaccharides, amino acids, proteins, lipids to more complex materials such as pesticides [9]. Diverse bacterial genuses are adapted to develop in polluted soils with pesticides. These microorganisms synthesize enzymes involved in the hydrolysis of P-O, P-F, P-S and P-C bonds, which are found in a wide variety of organophosphate pesticides [10].

Imidacloprid [1-(6- chloro-3-pyridylmethyl)-2-nitroimino-imidazolidine (IMI)] has gained great attention as a synthetic insecticide that acts in a similar manner as nicotine and is now widely used for the control of pests on crops. It is a very toxic and hazardous compound and a pollutant of environmental concern because of its increased usage worldwide [11]. 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 application of more pesticide. Although there are potential sources of toxic hazards from IMI, such as the global increase in its use and its persistence in crops, vegetables and fruits and physical contact in pets, there are few investigations on the toxic effects of IMI exposure in microorganisms [12]. Therefore, the present investigation was undertaken to evaluate its toxicity to soil bacteria *Bacillus weihenstephanensis* with emphasis on biochemical parameters and growth. Further biodegradation of imidacloprid by soil isolate *Bacillus weihenstephanensis* was carried out.

2. MATERIALS AND METHODS

2.1 Chemicals

The imidacloprid technical grade used in the study was purchased from a local agricultural dealer in Hubli. Analytical Grade Biochemical's, culture media were prepared according to Bergey's Manual. The glass wares used in the experiments were from Borosil Company.

2.2 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 concentrations [13]. Imidacloprid tolerant colonies isolated during the laboratory and field studies conducted to determine the effects of imidacloprid on soil bacterial populations [14]. The bacterium was maintained at 4ºC on nutrient agar [15] and sub cultured every fortnight. Synthetic sewage medium (S-medium) was used as the medium for toxicity testing.

2.3 Preparation of Inoculum

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

2.4 Identification of Bacterial Isolate

The pure culture was grown on nutrient agar medium. Colonies were characterized by cultural, morphological and biochemical characters and 16s rDNA identification.

2.5 Experimental Procedures

Estimation of biochemical parameters were done by using standard protocols. Estimation of DNA and RNA were determined by diphenylamine and orcinol [16] methods respectively. Protein was estimated by Lowry [17] method and the glucose content was estimated by Anthrone method described by [18].

2.6 Growth

The concentration of cells was measured every 24 hrs using optical density (OD) at 600 nm [19].

2.7 Isolation and Characterization of Imidacloprid-Degrading Bacterial Strains

Bacterial isolate SP-03 showed maximum degradation of imidacloprid during preliminary study and was cultured with MSM (KH_2PO_4(0.2g), K_2HPO_4(0.8g), $MgSO_4.7H_2O$(0.2g),$CaSO_4$(0.1g), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.001 g), and $(NH_4)_2SO_4$ (5.0g/100 mL)) and TSB (Pancratic digest of casein 17.00g, Papaic digest of soyabean meal 3.00g, Nacl 5.00g, Dipotassium hydrogen phosphate 2.50g and dextrose 2.50g) broth containing 10^{-3} molar imidacloprid and incubated at 37°C at 120 rpm for 28 days samples were taken at 0, 3, 7, 14 and 21 days and subjected to HPLC analysis to check the concentration of imidacloprid in the broth.

2.7.1 HPLC analysis

Periodic analyses of imidacloprid concentrations were accomplished using a Waters HPLC (Division of Millipore, Milford, MA), which had a Reverse Phase C-18 (RP18) Symmetry Shield column (Waters-Millipore) (3.9 mm x150 cm) and an ultra violet (UV) detector.

2.8 Plasmid curing of *Bacillus weihenstephanensis*

The LD-50 values were determined using a curing agent and then the cultures were subjected to plasmid curing.

2.8.1 Determination of LD-50

The Brevundimonas Sp. MJ 15 cultures was grown in different concentrations of acryflavin (0-50μg/ml) for 24h in nutrient broth and the OD of biomass were observed at 660nm against autoclaved media as blank. The OD of cultures was compared with control OD of culture (culture grown in absence of acryflavin) and the concentration of acryflavin giving nearly 50% less OD was considered as LD-50.

2.8.2 Procedure for plasmid curing

25ml of nutrient broth was prepared, autoclaved and inoculated with test organism and incubated at 37ºC for 18 hrs. 5 tubes of nutrient broth containing LD-50 concentrations of acryflavin were prepared and inoculated with 18hr old test organism (1%) in tube no.1. The control tubes without acryflavin were also prepared and inoculated, as mentioned earlier. The tubes were incubated at 37ºC for 24hrs. From tube no.1, 1% culture was inoculated to tube no. 2 and incubated further for 24hrs. This serial inoculation was continued for 5 generations. At every generation, the plasmid was isolated and run on 1% agarose gel to observe for the presence/absence of plasmid and simultaneously the sample was analyzed by HPLC for imidacloprid degradation.

2.9 Statistical Analysis

Statistic significance between the control and experimental data were subjected to analysis of variance (ANOVA) followed by post –hoc dunnet's test (P≤ 0.05).

3. RESULTS AND DISCUSSION

3.1 Identification of Soil Isolate

The bacterial strain SP-03 isolated from soil was a rod-shaped, Gram-positive bacterium, facultatively anaerobic, grows at 5-40ºC, 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. 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 22ºC and at pH of 7.0.

3.2 Effect of Imidacloprid Treatment on Bacillus weihenstephanensis

On exposure of *Bacillus weihenstephanensis* to various molar concentrations (10^{-3} to 10^{-7}) of imidacloprid for 24, 48, 72 and 96 hrs there was a significant (P≤0.05) decrease in the concentration of all the biochemical parameters studied. There was a significant decrease (P≤0.05) in the level of DNA (Fig. 1), RNA (Fig. 2), protein (Fig. 3) and glucose (Fig. 4) content in all the treated groups. There was a significant (P≤0.05) decrease in the growth in all the treated groups with an increase in dose and durational exposure to imidacloprid (Fig. 5).

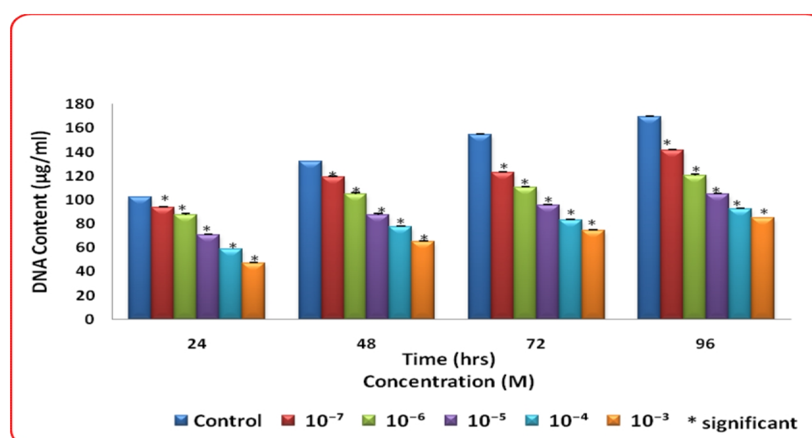

Fig. 1. Effect of imidacloprid on DNA content in *Bacillus weihenstephanensis*

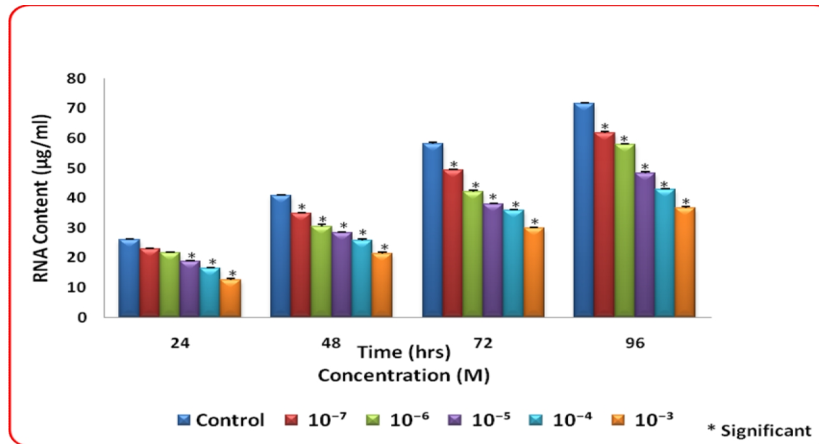

Fig. 2. Effect of imidacloprid on RNA content in *Bacillus weihenstephanensis*

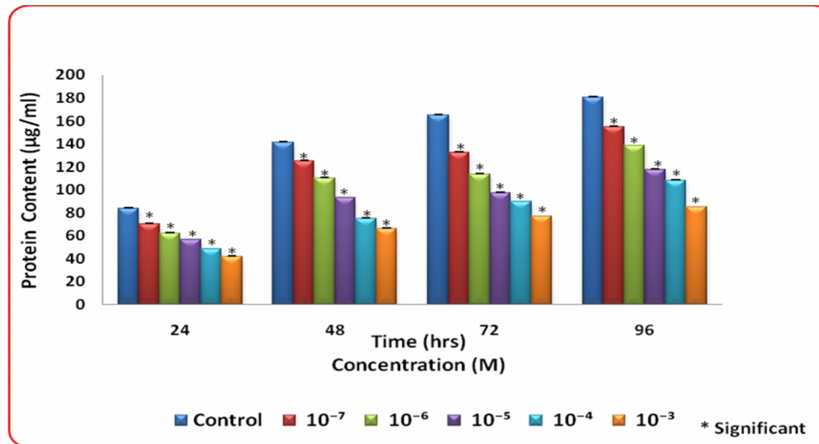

Fig. 3. Effect of imidacloprid on protein content in *Bacillus weihenstephanensis*

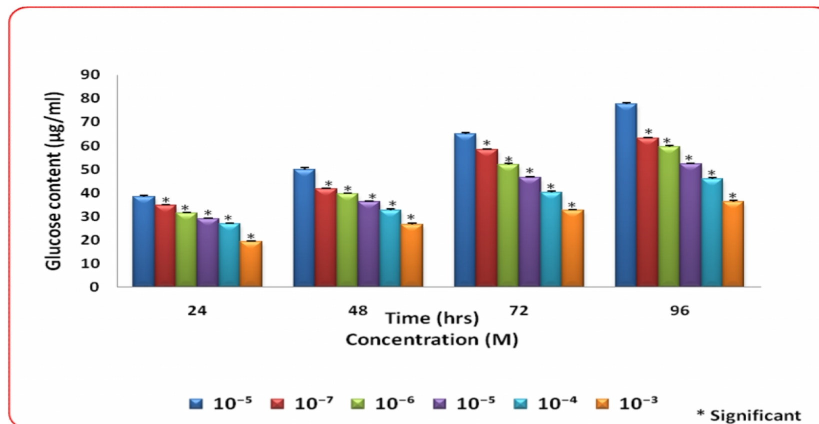

Fig. 4. Effect of imidacloprid on glucose of *Bacillus weihenstephanensis*

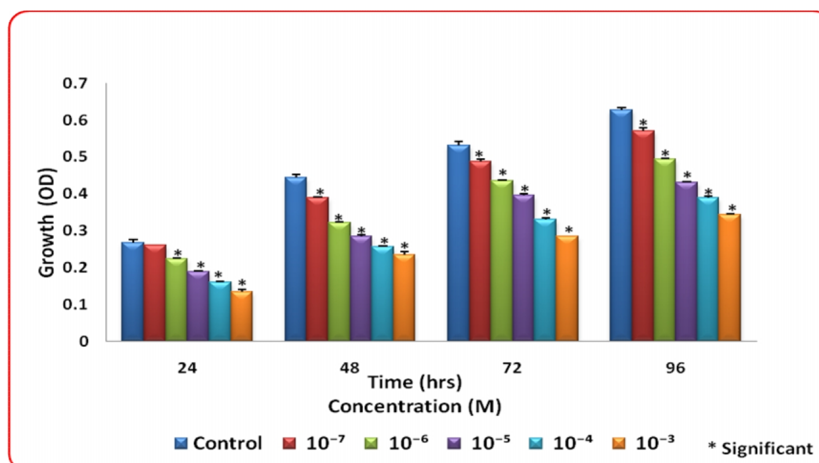

Fig. 5. Effect of imidacloprid on growth of *Bacillus weihenstephanensis*

3.3 Imidacloprid Degradation by Soil Isolate *Bacillus weihenstephanensis*

In the present study on treatment with 10^{-3} M of imidacloprid the degradation of imidacloprid in MS medium was 85, 76, 68 and 54% at a given duration of 3, 7, 14 and 28 days respectively from an initial concentration of 100%. In tryptic soya broth was 73, 57, 44 and 22% at a given duration of 3, 7, 14 and 28 days respectively from an initial concentration of 100% (Fig. 6). In the present study it was observed that, there was a significant decrease in the imidacloprid content in all the treated groups when compared with that of the controls. There was an appearance of peak during HPLC analysis which was confirmed as 6-chloro nicotinic acid based on the retention time at 219nm.

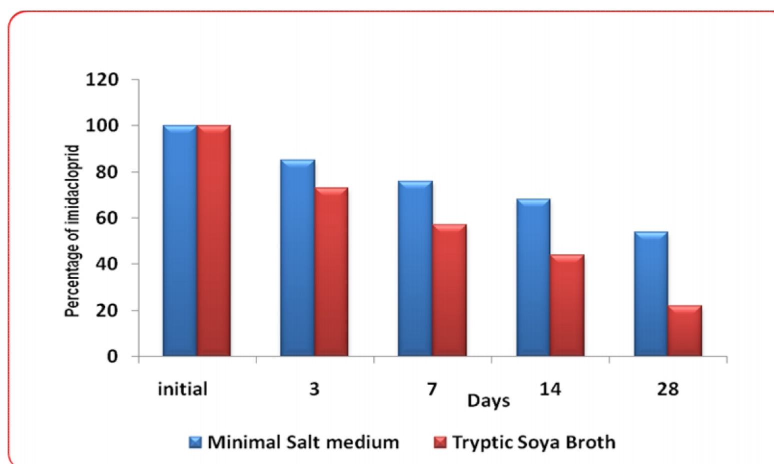

Fig. 6. Biodegradation of imidacloprid by *Bacillus weihenstephanensis*

3.4 Plasmid Curing

The size of the plasmid was estimated on the basis of electrophoretic mobility of the isolated fragments as compared to the sizes of marker (Fig. 5). The isolated plasmid DNA pattern of *Bacillus weihenstephanensis* showed that our strain harbors a plasmid. In the present study the LD-50 values obtained for *Bacillus weihenstephanensis* was 30 µg it was observed that the plasmid was cured in the fourth generation

The normal and plasmid cured strains were analyzed for imidacloprid degradation and it was observed that the normal cells of *Bacillus weihenstephanensis* showed a degradation of 75, 60, 45 and 25% of imidacloprid on the other hand the plasmid cured cells reported a degradation of 96.30, 87, 80 and 71.20% at 03, 07, 14 and 28 days respectively. The cured strain was able to degrade imidacloprid about 18.80% (Fig. 7) which indicates that the derivative genes are encoded by both chromosomal and plasmid DNA in *Bacillus weihenstephanensis*.

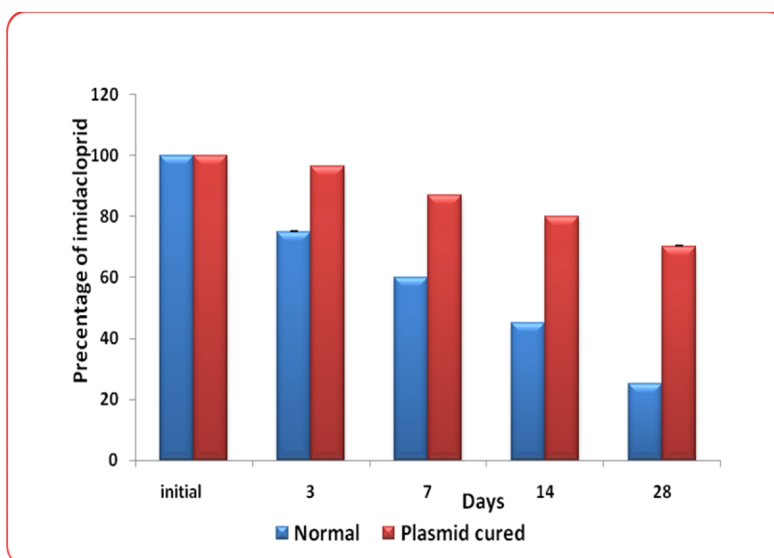

Fig. 7. Biodegradation of imidacloprid by normal and plasmid cured *Bacillus weihenstephanensis*

The increased use of pesticides in agricultural for pest control causes the contamination of soil with toxic chemicals. When pesticides are applied, the possibility exists that these chemicals may exert certain effects on non-target organisms, including soil microorganisms. The microbial biomass plays an important role in the soil ecosystems where they fulfill a critical role in nutrient cycling and decomposition. Pesticides may affect the microbial population by controlling the survival and reproduction of individual species.

The results of present study showed significant ($P \leq 0.05$) decrease in the DNA concentration and effect was dose and duration dependent. Pesticides like dicofol, carbosulfan, mancozeb, methomyl and indoxacarb caused decrease in DNA concentration in treated laborotary animals [20-22]. Studies on fresh water catfish, Clarias batrachus reported inhibitory effects of fenvalarate on DNA, RNA and protein content. This result showed extreme toxicity of pesticides on the main biochemical machinery of the cell. The

secretion of extra cellular proteins, including toxins and cellular effectors, is one of the key contributing factors in a bacterium's ability to thrive in diverse environments conditions. The decline in protein level indicates the physiological adaptability to compensate for pesticide stress and to overcome the stress; they use more energy, which leads to stimulation of protein catabolism. Similar results were reported in *Escherichia coli* and *Pseudomonas aeruginosa* exposed to various concentration of methomyl [13].

Proteins have many forms in the cell as structural proteins, enzymes, and functional proteins. Enzymes are important component of cell activity, as they catalyze all cellular reactions. Decrease in concentration of protein may be due to the toxicity and mutagenicity of the pesticide it is been reported that in rats treated with BHC caused significant reduction in hepatic DNA and RNA, with an indication of cell death due to focal necrosis. Rapid loss in proteins of the brain during pesticide toxicity was also reported [23, 24]. In a study on kidney biochemical contents showed increasing duration exposure of carbosulfan caused decrease in level of DNA, RNA, protein, glycogen, where as cholesterol increased significantly in male and female mice. Further a significant ($P \leq 0.05$) decrease in protein and glucose contents in treated groups observed in the present study may be due to the fact that the major protein modification is observed due to stress and the loss of catalytic activity, amino acid modification, carbonyl group formation, increase in acidity, decrease in thermal stability, change in viscosity, fluorescence, fragmentation, formation of protein- protein crosslink's, s-s bridges and increased susceptibility to proteolysis [25]. It has been reported that the biological targets for the reactive oxygen species due to oxidative stress are RNA, DNA, proteins and lipids.

The significant ($P \leq 0.05$) decrease in the growth observed in the present study may be due to the fact that imidacloprid might have affected the bacterial growth via possible attack to the membrane components and inhibited activity of DNA polymerase I [26]. The increase in percent inhibition in growth with increase in dose and duration of exposure of imidacloprid in cells is obligatory since some microbial groups will be able to use an applied pesticide as a source of energy and nutrients, where as others may well be toxic to other organisms and as such the soil microbial community is a complex picture of interwoven relationships between organisms in different tropic levels [27].

Soil isolate *Bacillus weihenstephanensis* degraded 46 and 78% imidacloprid in MSM and TSB respectively (Fig. 6). The pesticides once in environment cause many issues like health hazards to human's negative effect on wild and domestic animals. To overcome these problems in recent years many studies were under taken to isolate and characterize pesticide degrading microorganism from soil. A gram-negative bacterium *Achromobacter sp*. strain WM111 was isolated from an agricultural soil and was able to hydrolyze carbofuran insecticide [28]. *Bacillus sp.* are known to degrade xenobiotics and have fast growth at lower temperature and the degradation depends on the inoculum size also, though we have not studied the parameters influencing the biodegradation of imidacloprid it can be said based on literature available that lower temperature and neutral pH influence higher degradation of xenobiotics by *Bacillus Sp.* The *Bacillus licheniformis* strain isolated from the intestine of *Labeo rohita* by an enrichment technique showed capability of utilizing dimethoate as the sole source of carbon with the help of plasmid [29].

It is suggested that the detoxification metabolism occurs when a microorganism uses the pesticide as a carbon and energy source and the process is facilitated by resistant microorganisms. In general, the impact of pesticides on soil microflora is variable and results not only from the reaction of microorganisms to an active substances and formulation

additives but also from the development of specific group of microorganisms [30]. Some microbial groups are able to use an applied pesticide as a source of energy and nutrients to multiply [27], while there are some agrochemicals which are not utilizable by soil microflora and might be degraded in soil by microorganisms through co-metabolism [31-32]. The degradation of pesticides takes place by secretion of specific enzymes encoded in plasmid or chromosome of bacteria.

The size of the plasmid was estimated on the basis of electrophoretic mobility of the isolated fragments as compared to the sizes of marker (Fig. 8). The isolated plasmid DNA pattern of *Bacillus weihenstephanensis* showed that our strain harbors a plasmid. The plasmid curing experiments showed that the plasmid was lost after third generation of treatment with acryflavin (30µg/ml). The normal and plasmid cured strains were analyzed for imidacloprid degradation and it was observed that the normal cells of *Bacillus weihenstephanensis* showed a degradation of 75% on the other hand the plasmid cured cells reported a degradation of 18.80% respectively in 28 days of incubation. In the present study it was observed that cured strain acquired the ability to further degrade imidacloprid. Observing the HPLC results, it can be deduced that the gene responsible for bioremediation process was not exclusively encoded in the plasmid alone and that multiple genes present both on the plasmid and the main genome of *Bacillus weihenstephanensis*.

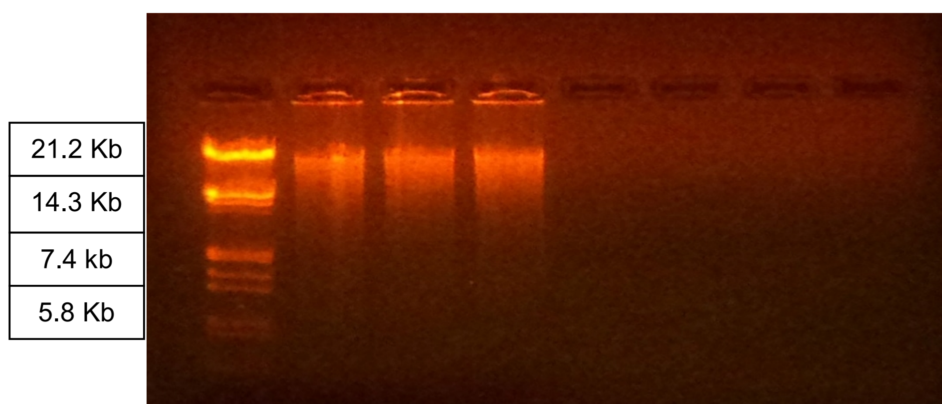

Fig. 8. Agarose gel electrophoresis of plasmid curing in *Bacillus weihenstephanensis* Lane 1 - Marker, Lane 2 – First generation, Lane 3 – Second generation, Lane 4 – Third generation, Lane 5 – Fourth generation, Lane 6 – Fifth generation

Several bacteria belonging to genus *Bacillus* have shown the ability to degrade pesticides. Three isolates namely, *Bacillus subtilis, Alcaligenes eutrophus* and *Pseudomonas aeruginosa* were obtained from the microcosms after successive enrichment. All the isolates grew readily on 100ppm of Aroclor 1221 concomitant with production of yellow metabolites in mineral salts medium [30]. *Bacillus* and *Psuedomonas sp.* isolated from groundnut field degraded Phorate, dichlorvos, methyl parathion, chlorpyrifos and methomyl [12]. The *Bacillus licheniformis* strain isolated from the intestine of *Labeo rohita* by an enrichment technique showed capability of utilizing dimethoate as the sole source of carbon with a help of plasmid [29]. *Pseudomonas stutzeri* and *Bacillus pumilis* enhanced carbofuran degradation, resulting in more than 98% loss of the applied carbofuran in 30 days [33]. Aerobic dieldrin- and endrin-degrading bacteria *Pseudomonas* sp., *Bacillus* sp., *Trichoderma viride* were isolated from soil [34]. One linuron-degrading isolate was identified as a *Bacillus*

sphaericus strain [35]. *Bacillus* sp. capable of metabolizing endosulfan was isolated from cotton-growing soil and effectively shown to degrade endosulfan into endosulfan sulfate [36].

These results indicate that not all the genes for degradation are located in plasmid but few are located on bacterial chromosome. In studies involving methomyl degradation by *Escherichia coli* similar results were reported in the same study plasmid of *Pseudomonas aeruginosa* had encoded all the genes required for methomyl degradation [13]. There are many reports of plasmid mediated degradation of pesticides. Soil isolate *Arthrobacter* was able to utilize and harbored three plasmids (designated pRC1, pRC2, andpRC3). These plasmids when introduced thought conjugation into non degrading mutants they transferred the degradation properties to these mutants, indicating that plasmids encoded all the genes required for carbaryl utilization [37]. Six independently isolated plasmids encoding the genes for degradation of the herbicides 2, 4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid are reported [38].

4. CONCLUSION

The present investigation indicates that the imidacloprid has a negative effect of biochemical parameters and growth of soil isolate *Bacillus weihenstephanensis* The *Bacillus weihenstephanensis* was able to degrade imidacloprid in minimal salt medium and tryptic soya broth. The soil isolate was able to utilize imidacloprid as sole carbon and nitrogen source as indicated by its growth in MSM. The soil isolate *Bacillus weihenstephanensis* hourberd a plasmid and it is involved in degradation of imidacloprid. The role of plasmid in degradation was proven by the curing. The curing also proved that not all the genes for degradation are encoded by plasmid but some are located in bacterial chromosome. Further one has to study the secondary metabolites for comprehensive knowledge of the degradation pathway. However, in order to judge the overall long-term effects of imidacloprid application on soil microorganisms it is necessary to carryout extensive studies on it effect to different group of microorganisms in soil.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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