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BIODEGRADATION OF IMIDACLOPRID BY SOIL ISOLATE *BREVUNDIMONAS SP*. MJ15

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ABSTRACT

Imidacloprid (I - [(6-chloro-3-pyridinyl)-methyl]-N-nitro-2-imidazolidinimine), is a chloronicotinyl insecticide used to control biting and sucking insects. Enrichment cultures from soil samples were made to isolate imidacloprid degrading bacteria. Soil isolates were tested for their ability to degrade imidacloprid in minimal salt medium (MSM) and tryptic soya broth (TSB). Further plasmid was isolated and curing was performed with acryflavin to establish the role of plasmid in degradation. Two soil-free stable enrichment cultures imi-01 and imi-05 were obtained. Strain SP-01 isolated from the enrichment cultures degraded 38 and 69 % of imidacloprid in MSM and TSB respectively in four weeks as confirmed by HPLC analysis at 0, 7, 14, and 28th day of incubation to determine imidacloprid concentration. Soil isolate SP-01 was identified as *Brevundimonas Sp.* MJ 15 by morphology, biochemical characters and 16S rDNA sequencing. Further a plasmid was detected in *Brevundimonas Sp.* MJ 15 and was cured in third generation. The cured cells showed 14.80% degradation compared to 58% degradation shown by non cure cells in TSB. Present investigation revealed that *Brevundimonas Sp.* MJ 15 was able to degrade imidacloprid in MSM and TSB. Further plasmid curing revealed that the genes responsible for imidacloprid degradation are located both in plasmid and chromosome.

INTRODUCTION

Various insecticides to protect crops against insects have been used over the last decades. They were applied by spraying in large quantities, thus inducing pollution of air, soils and waters. In the 1990s, new insecticides were sold and announced as being efficient. Their implementation allowed the reduction in use of large quantities of pesticides and thus, to reduce pollution. Imidacloprid is one of these new insecticides having molecular formula $C_9H_{10}Cl\ N_5O_2$ with a molecular weight of 255.7 g/mol. In appearance, it consists of colorless crystals (Placke and Weber 1993). It is a member of a relatively new class of insecticidal chemistry, the chloronicotinyl neonicotinoid compounds (Elbert *et al*., 1990). It 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. It is most commonly used on rice, cereal, maize, sunflowers, potatoes and vegetables. It is especially systemic when used as a seed or soil treatment (Nauen *et al*., 1998). The active chemical works by interfering with the transmission of stimuli in the insect's nervous system. Specifically, it causes a blockage in the nicotinergicneuronal pathway that is more abundant in insects than in warm-blooded animals, making the chemical much more toxic to insects than to warm-blooded animals. This binding on the nicotinic acetylcholine receptor (nAChR) leads to the accumulation of the acetylcholine neurotransmitter, resulting. In the paralysis and death of the insect (Okazawa *et al*., 1998). Xenobitics in soil are treated by bioremediation using microorganisms. This method is used successfully in many countries (Ritmann *et al*., 1988; Enrica, 1994). This method is used mainly because of it is eco-friendliness and cost effectiveness compared to physical and chemical remediation methods (Saaty and Booth, 1994). Bacterial species like *Peudomonas, Flavobacterium, Arthrobacter, Xanthobacter e*tc. have been isolated form soil for their capability to degrade pesticides and are used in bioremediation of different pesticides (Gossel and Bricker, 1992; Greer *et al*., 1992; Ishaq *et al*., 1994). Field studies showed that imidacloprid can persist in soil, with a half-life ranging from 42 to 129 days. Half-life in soil varies depending on soil type, use of organic fertilizers, and presence or absence of ground cover (Scholz *et al*., 1992). It is found that imidacloprid degraded more rapidly under vegetation, t1/2 48 days, versus 190 days without vegetation.

Degradation on soil via photolysis has a t1/2 of 39 days. In the absence of light, the longest half-life of imidacloprid was 229 days in field studies and 997 days in laboratory studies (Miles Inc., 1993). Biodegradation of pesticides is controlled by the availability of the pesticide to a pesticide degrading microorganism and the activity of the microorganism. Bio availability may limit the biodegradation of imidacloprid and its metabolites in soil, resulting in the long half-lives observed. Imidacloprid and its metabolites become increasingly strongly, bound to soil over time (Cox *et al*., 1997). Sorption of imidacloprid and its metabolites, tend to increase with increasing soil organic carbon (OC) content. As organic

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compounds age in soil, bioavailability has been shown to be reduced and degradation limited (Solecki *et al*., 1995). The strong sorption of aged imidacloprid residues in soil may make biodegradation of the aged imidacloprid residues difficult. The biodegradation of various N-containing heterocycles, including pyridine and nicotine, by isolated microorganisms has been described, but there are no reports of microbial degradation of imidacloprid. Therefore, the present investigation was under taken to isolate imidacloprid degrading bacteria from soil and to study the microbial degradation of imidacloprid.

MATERIALS AND METHODS

Chemicals

The imidacloprid technical grade used in the study was purchased from a local agricultural dealer store 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.

Soil sampling

The experiment was carried out during summer of 2011 at laboratory of Department of Biotechnology and Microbiology Karnatak University, Dharwad Karnataka State India. The black cotton soil samples (pH 7.8, 0 .9% OC, and 0.3% N) were collected from cotton fields around Hubli city. These fields had a history of imidacloprid applications. Black cotton soil was collected at a depth of 16 cm from four different spots in each plot and mixed together to get one sample. These soil samples were placed in sterile polythene bags separately and transferred to laboratory. Soil samples were passed through a sieve of 2 mm to remove stones and plant debris and were stored at 4 ° c until further use.

Enrichment cultures

The soil samples were used to create enrichment cultures for isolation of imidacloprid degrading bacteria. Three versions of Kaufman and Kearney's minimal salts medium (MSM) were prepared. Fifty ml C-limited MSM with 100 mg L' imidacloprid as the sole carbon source, 50 ml N-limited MSM with imidacloprid as the sole nitrogen source and sodium citrate and sucrose added as supplemental carbon sources, and 50 ml MSM broth, which contained all components plus sodium citrate, sucrose, and imidacloprid were inoculated with 2 g of soil to enrich for imidacloprid degrading microorganisms. Cultures were maintained on the shaker at 120 rpm at 37°c. Subcultures were made monthly in order to obtain soil-free enrichments. Aliquots of the cultures were periodically removed from the shaker flasks for HPLC analysis.

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.

Operating conditions were

30 min gradient of acetonitrile (ACN)and acidified (pH 3) ultrapure water [(0 mm) 20%/80%ACN:H2O; (7 mm) 22%/78% ACN:H70; (14 mm)30%/70°/ ACN:H2O; (21 mm) 40%/60% ACN:H20; (23-30 mm) 20%/80°A ACN:H20)], injection volume of 25 pl, flow at 0.6 ml and UV detection at wavelength of 270 nm. Media samples were filtered (0.2 m) and diluted by half with methanol prior to analysis (Dai *et al*., 2006).

Isolation and characterization of Imidacloprid-degrading bacterial strains

Two mixed enrichment cultures from the cotton soil that showed losses of imidacloprid during incubation, Imda-01and Imda-05, were spread-plated using dilutions of 10^{-3} to 10^{-6} on N-limited agar plates containing 50 mg kg' imidacloprid and cyclohexamide. After one week of incubation at 37°c, the plates were screened for colonies that visually appeared different from one another. In total, 06 colonies (SP-01 to 06) were transferred on to new agar plates with imidacloprid as the sole nitrogen source. After one week, SP strains 01-06 were put into 25 ml of tryptic soy broth (TSB) and after three days growth, each of the 06 isolates were centrifuged for 10 min at 6700 x g. The supernatant was poured off, and the isolates were re-suspended in sterile phosphate buffer for a total volume of 10 ml. 2 ml sample of each isolate was inoculated into N-limited MSM, C-limited MSM, and TSB all containing 30 mg L' of imidacloprid in 25 ml total. Non-inoculated controls were also made by inoculating 2 ml of phosphate buffer into each of the media-filled flasks. All samples were wrapped in aluminum foil and placed on a shaker operated at 37°C and 120 rpm. The three non-inoculated controls were analyzed for imidacloprid concentration. After seven days of incubation, the 06 cultures were extracted by adding *25* ml of methanol to the flasks. The samples were then centrifuged at 6700 x g. Four ml aliquots were filtered through a 0.2 µm filter and analyzed on the HPLC to check imidacloprid concentration in each culture. Bacterial isolate SP-01 showed maximum degradation of imidacloprid during preliminary study and was cultured with TSB and MSM broth containing 10^{-3} molar imidacloprid and incubated at 37^0c at 120 rpm for 28 days samples were taken at 0, 3, 7, 14 and 28 days and subjected to HPLC analysis to check the concentration of imidacloprid in the broth culture. The imidacloprid degrading bacteria SP-01 was then identified morphologically, biochemically and genetically. DNA was isolated from the culture (Ausubel *et al*., 1994). Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel (figer-1). The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1305 bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database.

Plasmid curing of *Brevundimonas Sp. MJ 15*

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

Determination of LD-50

The *Brevundimonas Sp. MJ 15* cultures was grown in different concentrations of acryflavin (0-50µg/ml) for 24 h in nutrient broth and the OD of biomass were observed at 660 nm 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.

Procedure for plasmid curing

25 ml of nutrient broth was prepared, autoclaved and inoculated with test organism and incubated at 37^0C for 18 hrs. 5 tubes of nutrient broth containing LD-50 concentrations of acryflavin were prepared and inoculated with 18 hr 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 37^0C for 24 hrs. From tube no.1, 1% culture was inoculated to tube no.2 and incubated further for 24 hrs. 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.

RESULTS AND DISCUSSION

Pesticides constitute the key control strategy for pest management and have been making significant contribution towards improving crop yields. Currently, among the various groups of pesticides that are being used world over, Chloronicotinyl form a major and most widely used group. The chances of isolating microbial strains from polluted soils, with high ability to metabolize a particular xenobiotic are brighter (Feng *et al*., 1997). Hence, the soil samples were collected from areas where imidacloprid was used constantly. Two soil-free enrichment cultures, imi-01 and imi-05, were obtained from the soil that degraded imidacloprid after several months of exposure to the insecticide. Cultures with consistent ability to degrade imidacloprid were not obtained from the other soils. During enrichment with a xenobiotic compound, the natural selection of microorganisms which have been adapted to the presence of that xenobiotic and its rapid biodegradation are known to take place (Cullington and Walker, 1999). In these enrichment cultures, imidacloprid concentrations decreased by about 56% and 33%, respectively, from initial concentrations of 10^{-3} molar in a period of four weeks. Cell-free control cultures showed no imidacloprid loss. Samples of the imi-01 and imi-05 enrichments were plated on N-limited agar containing imidacloprid six pure colonies selected from these plates were tested for imidacloprid degradation, but only strain SP-01 consistently degraded imidacloprid. Biodegradation of imidacloprid by soil isolate SP-01 in MSM, C-MSM and N-MSM broth are given in Table 1. SP-01 was able to degrade 05, 20, 31, and 40% of imidacloprid in N-MSM after 03, 07, 14 and 28 days of incubation respectively. In C-MSM 06, 22, 33, 42 % imidacloprid was degraded after 03, 07, 14 and 28 days of incubation respectively and in MSM 05, 18, 29, 36 percent of imidacloprid was degraded after 03, 07, 14 and 28 days of incubation respectively. No degradation was observed in the non-inoculated controls. The experiment showed that SP-01 was able to utilize imidacloprid as nitrogen and carbon source. Further cells of SP-01 were identified as those of a rod, Gram-negative bacterium. By sequencing the 16S rDNA gene of SP-01 was isolated (Fig. 01) 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 *Brevundimonas*. The sequence of strain SP1 displayed the highest identity (100%) with the 16S rDNA gene of *Brevundimonas sp. MJ15* (GQ250440.2).

Degradation of imidacloprid by soil isolates

Bacterial isolate *Brevundimonas sp.* MJ15 (SP-01) was cultured with MSM and TSB broth containing 10^{-3} molar imidacloprid and incubated at 37 $^{\circ}$C at 120 rpm for 28 days .samples were taken at 0, 3, 7, 14 and 28 days and subjected to HPLC analysis to check the concentration of imidacloprid in the broth culture. Biodegradation of imidacloprid by soil isolate *Brevundimonas sp. MJ15* in MSM and TSB broth is given in Table 2. The imidacloprid concentration in TSB was 37, 48, 57 and 69% after 03, 07, 14 and 28 days of incubation respectively. The imidacloprid concentration in MSM broth decreased by 10, 19, 26, and 38% after 03, 07, 14 and 28 days of incubation respectively. The results indicate that Brevundimonas sp. MJ15 was able to degrade imidacloprid by 38 and 69% in 10^{-3} molar MSM and TSB respectively in 28 days of incubation. The discovery of microorganisms capable of tolerating or growing in high concentrations of pesticides provides a potentially interesting avenue for treating hazardous wastes (Guha *et al*., 1999). 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 (Topp*, et al*., 1993). 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 (Mandal *et al.,* 2005). Report of *Alcaligenes xylosoxidans* a Pentachloronitrobenzene degrading strain that is able to utilize and grow on Pentachloronitrobenzene as a sole carbon source was isolated (Eun-Taex *et al*., 2003). In another report most of the fenamiphos degrading bacteria's were *Microbacterium* species, although *Sinorhizobium, Brevundimonas, Ralstoniaand Cupriavidus* were also identified (Cabrera *et al.,* 2010). *Pseudomonas putida*, *P. aeruginosa* and *Acetobacter faecalis* respectively were able to degrade glyphosate even at high concentration (Olawale *et al*., 2008). Microorganisms have increasingly been investigated and *Fusarium ventricosum* degraded endosulfan up to 70% (Siddique *et al.,* 1997). Other than these pesticides bacteria's capable of degrading chlorpyrifos, thiaram, dicofol, cypermeritin, malathion, profenofos etc (Ghanem *et al.,* 2007; Kamal, *et al.,* 2008; Sankar, *et al.,* 2009; Sahin, 1999; Mohamed, 2009 Malghani *et al.,* 2009) are been isolated and these microbes are used to clear residues of these pesticides from soil. In our study the soil isolate *Brevundimonas sp. MJ15* was able to degrade imidacloprid and use it as nitrogen and carbon source as indicated by growth of bacteria and by decrease in imidacloprid concentration by 68 and 38% in MSM and TSB mediums respectively. In soil imidacloprid

Table 1. Biodegradation of imidacloprid by soil isolate *SP*-01 in Minimal Salt Medium.

		Degradation (%)			
Group	*Initial concentration*	*Days*			
	(10^{-3} Molar)	*03*	*07*	*14*	*28*
Control	*100*	*98*	*96*	*95*	*95*
N-MSM	*100*	*95*	*80*	*69*	*60*
C-MSM	*100*	*94*	*78*	*67*	*58*
MSM	*100*	*95*	*82*	*71*	*64*

Table 2. Biodegradation of imidacloprid by soil isolate *Brevundimonas sp. MJ15 i*n Minimal Salt Medium and Tryptic Soya Broth.

		Degradation (%)			
Group	Initial concentration	Days			
	(10^{-3} molar)	03	07	14	28
Control	100	98	96	95	95
MSM	100	90	81	74	62
TSB	100	77	63	52	31

Table 3. Biodegradation of imidacloprid by normal and cured cells of *Brevundimonas sp. MJ15* in Tryptic Soya Broth.

		Degradation (%)			
Group	Initial concentration	Days			
	(10^{-3} molar)	03	07	14	28
Control	100	98	96	94	92
Normal cell	100	79	65.80	53.30	42.20
Cured cells	100	96	92	90	85.20

metabolites are reported, these metabolites may be produced due to co-metabolism by bacterial communities present in soil the metabolites reported are imidacloprid urea, 6-chloronicotinic acid and 6-hydroxynicotinic acid (Sarkar *et al*,. 2001).

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 (Nowak *et al*., 1999). Some microbial groups are able to use an applied pesticide as a source of energy and nutrients to multiply (Johansen *et al*., 2001), while there are some agrochemicals which are not utilizable by soil microflora and might be degraded in soil by microorganisms through co-metabolism (Bollag and Liu, 1990). The degradation of pesticides takes place by secretion of specific enzymes encoded in plasmid or chromosome of bacteria. In our study we isolated a plasmid from *Brevundimonas sp. MJ15* and further plasmid curing was carried out to check the involvement of the plasmid in imidacloprid degradation. The LD-50 value obtained in our result for *Brevundimonas Sp.* MJ 15 was 40 µg/ml.

Plasmid curing and imidacloprid degradation by *Brevundimonas sp.* MJ15

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. 1). The isolated plasmid DNA pattern of *Brevundimonas sp.* MJ15 showed that our strain harbors a plasmid. The plasmid curing experiments showed that the plasmid was lost after third generation of treatment with acryflavin (40 µg/ml). The normal and plasmid cured strains were analyzed for imidacloprid degradation is given in table 3 and it was observed that the normal cells of *Brevundimonas sp.* MJ15 showed a degradation of 21, 34.20, 46.70, and 57.80% on the other hand the plasmid cured cells reported a degradation of 4.0, 9.0, 10., and 14.80% at 03, 07, 14 and 28 days respectively.

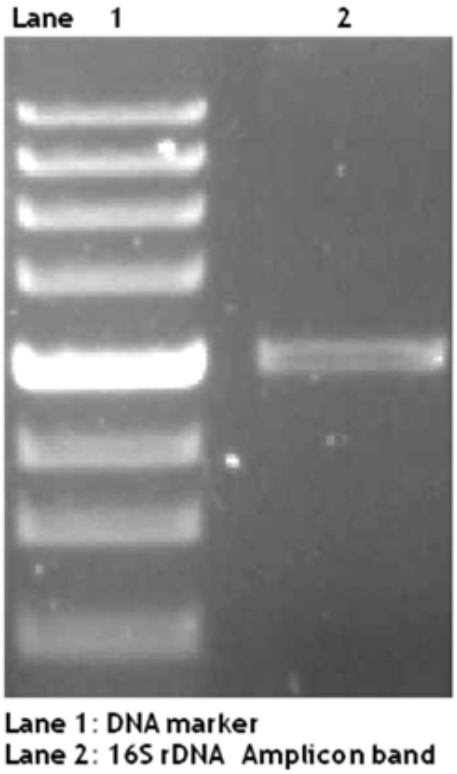

Fig. 1. Gel Image of 16S rDNA Amplicon of *Brevundimonas sp. MJ15* (SP-01)

In the present study it was observed that cured strain was 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 *Brevundimonas sp. MJ15*. Bacterial plasmid plays a role in the degradation of the

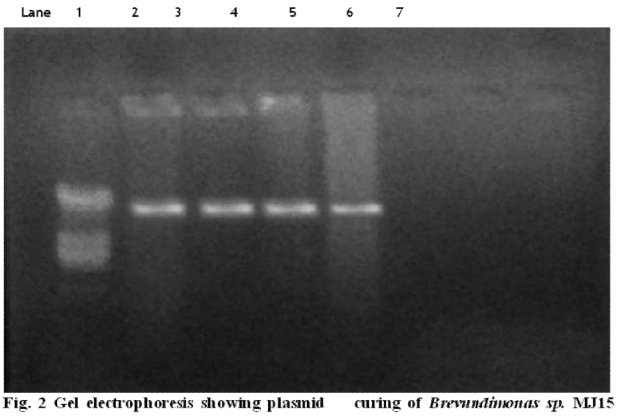

Fig. 2 Gel electrophoresis showing plasmid curing of *Brevundimonas sp.* MJ15 Lane 1: supercoiled DNA marker, Lane 2: Plasmid profile of Wild type *Brevundimonas sp. MJ15* Lane 3: Plasmid profile of *Brevundimonas sp. MJ15* after curing(first generation) , Lane 4: Plasmid profile of *Brevundimonas sp. MJ15* after curing(second generation) , Lane 5: Plasmid profile of *Brevundimonas sp. MJ15* after curing(Third generation), Lane 6: Plasmid profile of *Brevundimonas sp. MJ15* after curing (forth generation), Lane 7: Plasmid profile of *Brevundimonas sp. MJ15* after curing (fifth generation).

pesticide. The cured strain, in the present study, was able to utilize imidacloprid in TSB but at slower rate. The catabolic pathways which encode numerous aromatic hydrocarbon degradation pathways are frequently located on plasmids, although the pathways for some xenobiotic compounds such as chlorinated aromatic hydrocarbons and some pesticides can be located on either chromosome or plasmid. The widespread occurrence of bacteria with degradation abilities may be due to the spread of degrading genes among environmental microflora (Roy *et al*., 2002). In the present study, the evidence for a plasmid being involved in imidacloprid-degradation comes from the fact that the cured cells of *Brevundimonas sp. MJ15* were able to degrade imidacloprid but at slower rate than the normal cells and the concentration of imidacloprid remaining in broth after 28 days of incubation was 85.20% compared to normal cells which had only 42.20% remaining in broth. 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 (Kulkarni and Kaliwal, 2011). 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 (Hayatsu, *et al*., 1999). Six independently isolated plasmids encoding the genes for degradation of the herbicides 2, 4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid are reported (Don and Pemberton, 1999).

The *Pseudomonas* sp. Strain ZEA-1 was isolated from rhizosphere of corn plant by an enrichment technique showed capability of utilizing zearalenone as the sole source of carbon the transconjugant harbored a plasmid of the same molecular size (approximately 120 Kb) as that of donor strain, while cured strain was plasmid less and did not utilize zearalenone (Abdullah and Altalhi, 2006). In another study the ability of enzymatic degradation of dimethoate was plasmid mediated in *B. licheniformis, P. aeruginosa, A. hydrophila*, *P. mirabilis* and *B. pumilus* bacterial strains (Olawale *et al*., 2008). The present investigation revealed that soil isolate *Brevundimonas sp. MJ15* obtained after enrichment of soil sample was able to degrade imidacloprid in both MSM and TSB as indicated by decrease in concentration when compared to non-inoculated controls. The soil isolate *Brevundimonas sp*. MJ15 harbor a plasmid and the plasmid encodes some of the genes responsible for imidacloprid degradation as proven by curing experiment. In order to study degradation pathway it is necessary to identify metabolites of degradation and also to study the degrading enzymes in future.

Conclusion

Brevundimonas sp. MJ15 (SP-1) was enriched and isolated as imidacloprid degrading bacteria from agricultural soil with history of imidacloprid exposure. The *Brevundimonas sp.* MJ15 was able to degrade imidacloprid in minimal salt medium and tryptic soya broth containing 10^{-3} molar imidacloprid by 38 and 69% respectively as shown by periodic HPLC analysis for 21 days. The soil isolate was able to utilize imidacloprid as sole carbon and nitrogen source as indicated by its growth in C-MSM and N-MSM mediums and decrease in concentration of imidacloprid in 28 days of incubation. The soil isolate *Brevundimonas sp.* MJ15 hourberd a plasmid and it is involved in degradation of imidacloprid. The role of plasmid in degradation was proven by the curing. The cured cells of *Brevundimonas sp.* MJ15 were not able to degrade imidacloprid rapidly when compared to non cured cells as indicated by periodic HPLC analysis for 28 days. 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.

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