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Bioremediation of Hexaconazole by Soil Isolate- *Stenotrophomonas maltophilia*

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Amritha G. Kulkarni, Akshata Naik, Chetan Halenaik, Sahana Durgekar, Sheetal Naik, Shivakantkumar Adhikari, Sonali Chinchankar, A. B. Vedamurthy*

P.G. Department of Studies in Biotechnology and Microbiology,
Karnataka University, Dharwad – 580 003, INDIA.

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ABSTRACT

Continuous and extensive application of fungicides often has created environmental concerns due to their toxicity towards non-target organisms. Repeated application of hexaconazole may lead to accumulation, leaching, or affect the soil microorganisms. Its toxicity to man, mammals, fish and bird by oral, inhalation or contact route is very high. Hence, biodegradation of hexaconazole fungicide was carried out *in vitro* by strains isolated from the rhizosphere. A total number of eight strains were isolated and further screened based on their tolerance level to hexaconazole. Isolate No: 4 which showed maximum hexaconazole degradation was further sent for 16s rDNA identification. The selected isolate was gram negative bacilli, Indole, MR-VP, H_2S and Catalase +ve, and -ve for citrate utilization and starch hydrolysis. The 16s rDNA reports revealed that the accession number was NC 0109431 and the culture showed 99% similarity to *Stenotrophomonas maltophilia* The present study indicates that hexaconazole is moderately persistent in soil. The plasmid curing study confirmed that the genes of the plasmid of *Stenotrophomonas maltophilia* are involved in the hexaconazole degradation and such plasmids could be used in combination with recombinant DNA technology for bioremediation of pollutants.

INTRODUCTION

Hexaconazole fungicide, classified under the triazole group, prolongs the productive life of infected palms compared to other commonly used fungicides such as thiram, benomyl, triadimefon, triadimenol, and tridemorph (Idris *et al*., 2002). Triazole fungicides like hexaconazole, propiconazole, triadimefon and paclobutrazol have been extensively used on crops worldwide. Among these triazole fungicide hexaconazole (2-(2,4-dichlorophenyl)-l-(1H-l,2,5-triazol-l-yl). hexan-2-ol has been introduced by ICI Agrochemicals in early 1980s and is sold under the trade name of ANVIL and PLANETE (Smith, 1991). It is also applied in post-harvest food storage, as a seed pre-planting treatment and as a timber treatment fungicide (Tomlin *et al*., 2003). Previous studies showed that fungicidal treatments of hexaconazole were effective against Ganoderma disease in oil palm (Razak *et al*., 2004). Triazole fungicides are widely used on cereals and ornamental plants to control fungi particularly Ascomycetes and Basidiomycetes. Hexaconazole is the common name for (RS)-2-(2,4-dichlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol. It has systemic demethylation inhibitors (DMI) that act mainly on the vegetative stage of fungi, by blocking the mycelial growth either inside or on the surface of the host plant (Spiroudi *et al*., 1998). In Malaysia, hexaconazole has been recently introduced to control white root disease pathogens on a variety of crops (Lam and Chiu *et al*., 1993; Chia *et al*., 1997). However, some work has been published on degradation of hexaconazole in peaches and in model systems (Angioni *et al*., 2003), dissipation in vegetables (Han *et al*. 2013), and effect of bio-compost amendment (Singh and Dureja *et al*., 2009). Other published works have reported on the persistence of hexaconazole in several soil conditions (Singh and Dureja *et al*., 2000), sorption and leaching behaviour (Sharma *et al*., 2013), photodegradation of hexaconazole in solvent (Santoro *et al*., 2000), and leaching potential and mobility in soil (Singh 2005; Fenoll *et al*., 2010). In a previous work by (Halimah *et al*., 2012), the residues of hexaconazole were reported in oil palm plantation in soil and leaf. Such persistence of pesticide in soils may be attributed to its higher absorption on soil particles due to the hydrophobic nature of the fungicide (Kulkarni and Vedamurthy *et al*., 2015).

It has a broad spectrum activity against Ascomycetes and Basidiomycetes (Worthington *et al*., 1991). Its toxicity to man, mammals, fish and bird by oral, inhalation or contact route is low to moderate. The Canadian Pest Management Regulatory Agency, who has recently completed a toxicological evaluation, has recommended an acceptable daily intake of 0.005

mg kg'1 of hexaconazole in drinking water. The forest product industry uses several formulations of hexaconazole to control staining and decay fungi, as well as mold. The compound is also recommended for use on fruits, flowers, vegetables, plantation crops and cereals (Waller *et al*., 1990; Cotterill and McLean *et al*., 1992; Lam and Lim *et al*., 1993; Chia *et al*., 1997). During the development of new pesticides, environmental considerations require an understanding of how new agents may be distributed and persist in the environment. In soil, for example, xenobiotics may undergo chemical physical and/ or biological transformations, all of which control their environmental persistence. In general, triazole fungicides are characterised by their long persistence in soil (Patil *et al*., 1988; Bromilow *et al*., 1999. Atreya *et al*., (1990) has reported half-life of hexaconazole in Malaysian soils is up to 2 months and about 15% of it remains un degraded even up to six months. As there is little information available about the persistence and mode of dissipation of hexaconazole from tropical soils. The effect of temperature, moisture, sterilization and organic matter amendments on the persistence of hexaconazole in different soils. The quality of soils, groundwater, inland and coastal waters, and air are all affected by pesticide contamination (Hernandez and Salinas *et al*., 2010). Therefore, the aim is to study in-depth dissipation of hexaconazole in the plantations and to identify the factors responsible for the downward movement of hexaconazole in soil profile. Further, focusing towards the environmental and global solution by isolating and screening the most potential bacteria responsible for the degradation of hexaconazole.

MATERIALS AND METHODS

Fungicide: Hexaconazole:

Analytical grade hexaconazole (95% purity) was bought from Rallis India Ltd., Bangalore, India.

Isolation of hexaconazole degrading bacteria from soil sample

Soil sample was collected from the Agriculture University Dharwad campus, Dharwad, where the soya bean plants were grown and where the commercial hexaconazole was sprayed (5%). 2mg /ml of analytical of hexaconazole was dissolved in methanol. 1 gm of soil sample was added to 10 ml of autoclaved Minimal Salt Medium (MSM) containing hexaconazole (10^{-3}M) as a source of carbon and energy. Cultures were incubated in an orbital shaker at 37^{0}C 120 rpm for 24-48 hrs. After enrichment cultivation, 0.1 ml of soil sample was spread

on the Minimal Salt Agar plates (MSA) that contained hexaconazole as a sole carbon source. These plates were incubated at 37^0 C for 24-48 hrs. A luxuriantly grown colony from each isolate were than used to inoculate into 10ml of minimal salt medium containing hexaconazole and further incubated for 24 hrs in an orbital shaker under the same condition in order to confirm the biodegradability. These strains were subjected to High Performance Liquid Chromatography (HPLC) analysis**.** The isolated strain which showed maximum hexaconazole degradation was subjected and regularly subcultured to ensure the maintenance of the degradative phenotype.

Characterization and Identification of Isolated Bacterial Strain

The selected, isolated, most potential bacterial strain was characterized and identified on the basis of morphological and biochemical parameters. This was sent to Biogenics, Pvt India Ltd, where it was subjected to further molecular characterization by Polymerase Chain Reaction (PCR) amplification and sequence analysis by 16S rDNA identification

Determination of Hexaconazole (%) Using HPLC

Degradation of hexaconazole by the isolated strain was confirmed by high performance liquid chromatography (HPLC). The first one (Standard sample) was non-inoculated sample containing hexaconazole (10-3 M) in 100 ml of minimal medium was kept as a control. In the test sample, 5 ml of the culture was added to sterile minimal medium containing hexaconazole (10-3 M) incubated in orbital shaker at 120 rpm at 37^0C and the percentage degradation was analyzed every 24hrs up to 96hrs. HPLC analysis was performed at prajna Bioscience Pvt. Hubli, India. The analytical column used was C-18 (150 mm x 3 mm, 3 µm). The injection Volume was 20 µl. The isocratic elution conditions.Acetonitrile: 0.1% Formic Acid (70:30). The wavelength for detection was 240 nm.C-18, Flow rate 1ml/min

Plasmid Curing Of Soil Isolate:

Weigh 250mg of agarose and dissolve it in 25 ml of TAE buffer (4.84 g Tris base, pH 8.0, 0.05 M EDTA/1 ltr) by boiling. When the gel attains ~55^0C, pour it into the gel cassette fitted with comb. Let the gel to solidifyCarefully remove the comb, place the gel in the electrophoresis chamber flooded with TAE buffer. Load 20 µl of DNA sample (mixed with bromophenol blue dye @ 1:1 ratio), carefully into the wells, along with standard DNA marker and pass the constant 50 V of electricity for around 30 min Remove the gel and

carefully stain with ETBR solution (10 µg/ml) for 10-15 min and observe the bands under UV transilluminator.

The LD-50 values were determined using a curing agent and then the cultures were subjected to plasmid curing. The bacterial isolate 4 was grown in nutrient broth for 18 hours and inoculated (0.1 ml) into nutrient broth tubes (5 ml) with different concentrations of acridine orange (5-50µg/ml). A control tube without acridine orange was also maintained. The cultures were grown for 24 h at 37ºC and the biomass was measured at 660 nm. The OD values of treated samples were compared with that of control and LD-50 concentrations were determined. This was further subjected to curing.

25 ml 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 acriflavine were prepared and inoculated with 18 hr old test organism (1%) in tube no.1. The control tubes without acriflavine were also prepared and inoculated, as mentioned earlier. The tubes were incubated at 37ºC 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. The plasmid isolated from treated bacterial samples of *Stenotrophomonas maltophilia* were run on agarose gel and pictures are presented below. The isolate's plasmid was cured by 3rd generation.

RESULTS

Table 1: Morphological characteristics of soil isolates

Isolate No.	Shape	Margin	Elevation	Texture	Appearance	Colour	Optical Property	Gram's nature
1	Irregular	Entire	pulvinate	Rough	Dull	cream	Opaque	Gram+ve (Bacilli)
2	Irregular	Entire	Convex	Smooth	Shiny	cream	Opaque	Gram-ve (short rod)
3	Irregular	Entire	Convex	Smooth	Shiny	cream	Transluc ent	Gram+ve (Bacilli)
4	Irregular	Entire	Umbonate	Smooth	Shiny	cream	Opaque	Gram -ve (Bacilli)
5	Circular	Entire	pulvinate	Smooth	Shiny	cream	Opaque	Gram+ve (Bacilli)
6	Irregular	Curled	Convex	Smooth	Shiny	cream	Opaque	Gram-ve (Diplococci)
7	Irregular	Curled	Convex	Smooth	Shiny	cream	Opaque	Gram +ve (Streptobacilli)
8	Circular	Entire	Convex	Rough	Dull	Cream	Opaque	Gram -ve (Cocci)

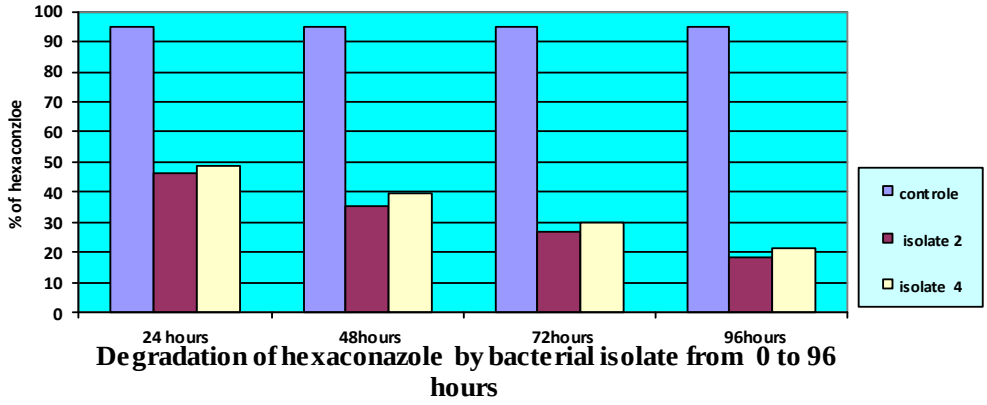
Table 2: Biochemical Characteristics of the Soil Isolates

Name of the Test	**Isolate:1**	**Isolate:2**	**Isolate:3**	**Isolate: 4**	**Isolate:5**	**Isolate:6**	**Isolate: 7**	**Isolate: 8**
Indole	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve
Methyl red	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
Voges-proskauer	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
Citrate utilization	-ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve
H_2S production	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Starch hydrolysis	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve
Urease	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve

Table 3: HPLC Analysis of Hexaconazole degradation by different isolates

Isolate no.	Initial Concentration of Hexaconazole (%)	Degradation of Hexaconazole in 24 hrs (%)
Control	95.04	95.04
1	95.04	60.67
2	95.04	67.37
3	95.04	48.78
4	95.04	69.08
5	95.04	64.47
6	95.04	46.17
7	95.04	59.35
8	95.04	65.97

Graph 1: Biodegradation of Hexaconazole by Bacterial Isolate No: 2 and 4



Graph 2: Chromatograph of *Stenotrophomonas maltophilia*

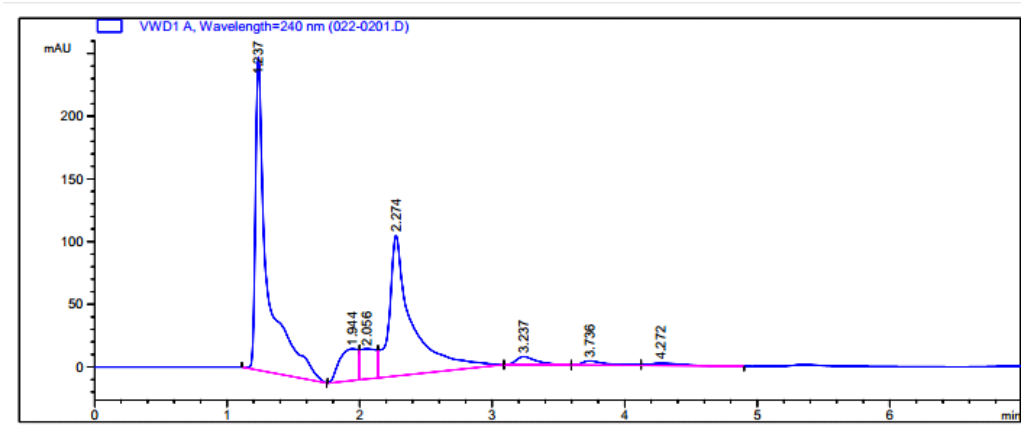


Figure 1: Colony morphology of *Stenotrophomonas maltophilia*

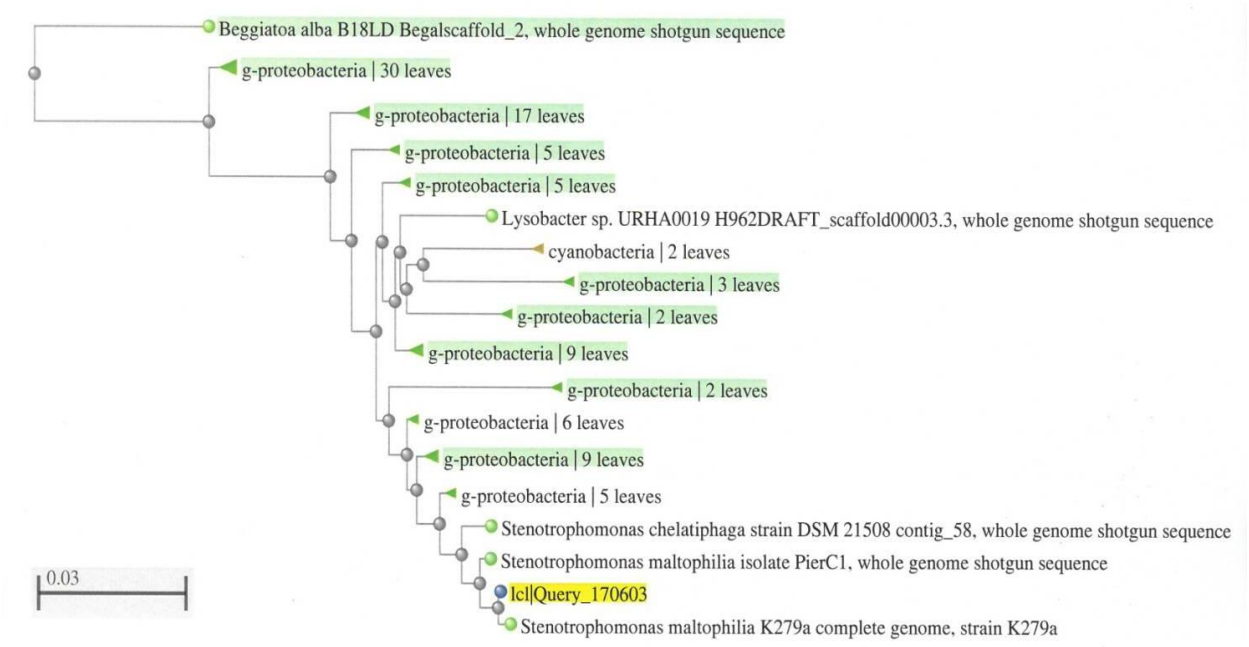


Figure 2: Phylogenetic tree of *Stenotrophomonas maltophilia*

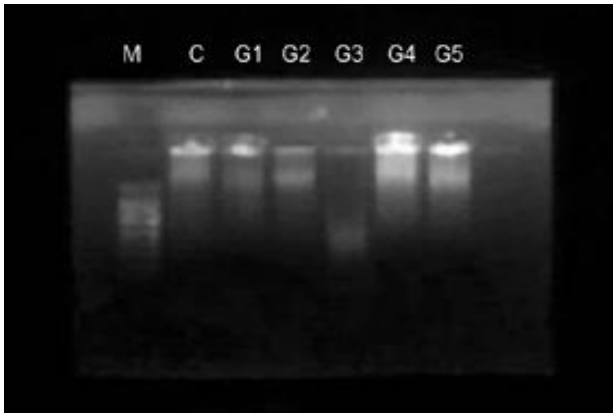


Figure 3: Plasmid Curing of *Stenotrophomonas maltophilia*

M- Marker, C- Control, G1- 1st generation, G2- 2nd generation, G3- 3rd generation, G4- 4th generation, G5- 5th generation

DISCUSSION

Soil samples were collected from Agriculture University, Dharwad campus, Dharwad, of which 8 isolated were observed. Morphological characteristics of the isolates were studied (Table 1). All the isolates were subjected to HPLC analysis and the degradation of hexaconazole (%) observed for the Isolates No. 1,2,3,4,5,6,7,8 and were 60.7,46.17,69.08,48.78,64.47,67.37,59.35and 65.97 % respectively, from the initial control of 95.04 %. The present study revealed that there was a significant degradation of hexaconazole, observed with all the isolates. However, maximum degradation of hexaconazole was observed by the isolate No.4. The Isolate No-4 (Fig. 1) which showed maximum hexaconazole degradation was further sent for 16s rDNA identification. The selected isolate was gram negative bacilli, the biochemical characteristics showed). It is Indole, MRVP, H_2S and Catalase +ve, and Negative for citrate and starch hydrolysis (Table:2). The 16s rDNA reports revealed that the accession number was NC 0109431 and the culture showed 99% similarity to *Stenotrophomonas maltophilia* (Fig. 2 and 3)

The discovery of microorganisms capable of tolerating or growing in high concentrations of pesticides provides a potentially interesting avenue for treating hazardous wastes (Mandal and Pal, 2005). Some investigations resulting in the identification of microbial isolates which are apparently responsible for the accelerated degradation of individual pesticides is necessary (Chaudhry and Wheeler *et al*., 1988; Racke and Coats *et al*., 1988,) of which the 16s rDNA analysis is considered to the most valid and widely used technique in the identification of microorganisms (Borich and Fulekar *et al*., 2009)

The present study revealed that the Hexaconazole was decreased significantly by *Stenotrophomonas maltophilia*. However different degradation rates were observed. All the organisms could utilize Hexaconazole as a sole carbon source and had the bioremediation ability. Further it was observed that the soil isolate *Stenotrophomonas maltophilia* exhibited a good growth rate and higher biodegradation efficiency towards Hexaconazole, and compared to the other isolate it is suggested that the detoxification metabolism occurs when a microorganism uses the pesticide as carbon source (Mohamed *et.al*, 2009) earlier studies have reported that the isolated *Stenotrophomonas maltophilia* M1 strain possesses a strong

ability for hexaconazole degradation. This strain contains a plasmid which was believed to be responsible for the degradation of hexaconazole carrying the degrading gene. Such plasmid could be transferred to another bacterial strain in the environment and provide it with additional pesticide degradative ability and potentially selective advantage under a given environmental state (Kulkarni and Kaliwal *et.al*, 2016).

Stenotrophomonas maltophilia is an uncommon, aerobic, non-fermentative, gram negative bacterium; motile due to polar flagella, catalase-positive, oxidase-negative .which distinguishes them from most other members of the genus and have a positive reaction for extracellular DNase. While S. maltophilia is an aerobe, it can still grow using nitrate as a terminal electron acceptor in the absence of oxygen (Crossman *et al.*, 2008). *S. maltophilia* strains are found to be ubiquitously distributed in the environment with regard to habitat and geography: often associated with roots of many plant species (Ryan *et al*., 2009). Growth of *S. maltophilia* studied in presence of different carbon sources: trichloroethylene (TCE), toluene, phenol, glucose, chloroform, and benzene with 0.1% peptone revealed an interesting growth pattern. Growth in presence of TCE, benzene, and chloroform was almost the same, whereas comparatively less growth was seen in presence of toluene and no growth in phenol even in presence of peptone (Mukherjee and Roy, 2013c). Xenobiotic-degrading *S. maltophilia* have tremendous potential for bioremediation but new modifications are required to make such microorganisms effective and efficient in removing these compounds, which were once thought to be recalcitrant. Metabolic engineering through genomic manipulations might help to improve the efficiency of degradation of toxic compounds by *S. maltophilia.* However, efficiency of naturally occurring *Stenotrophomonas* sp. For field, bioremediation could be significantly improved by optimizing certain factors such as bioavailability adsorption and mass transfer. Chemotaxis and microbe–plant interactions could also have an important role in enhancing biodegradation of pollutants (Kulkarni and Kaliwal *et.al.*, 2015).

On treatment with hexaconazole, the degradation of methomyl was 48.78, 39.35, 23.45, and 16.18% at a given duration of 24, 48, 72 and 96 hrs respectively from an initial concentration of 95.04 %. The results of the present study revealed that there was a significant decrease in the hexaconazole content in the treated group when compared with that of the control of *Stenotrophomonas maltophilia*. The plasmid curing results in the present study reveal that the degrading ability for Hezaconazole is encoded in the plasmid for Stenotrophonomas maltophilia. Many xenobiotic degradation genes present in soil bacteria have been shown to

reside on plasmid, a common location for other degradation genes (Altalhi *et.al.,* 2007). Soil microbial populations, particularly members of the genus Pseudomonas, have evolved the considerable nutritional versatility and are capable of the degradation of a range of complex, naturally occurring aromatic and aliphatic compounds an interesting feature of these degradative plasmids are that they have been isolated almost exclusively from *Pseudomonas aeruginosa* (Kulkarni and Kaliwal *et.al.,* 2015). Significantly these plasmids have been isolated most exclusively from species of the genus Pseudomonas, a group of bacteria known to play a major role in the breakdown and recycling of organic molecules both in soil and water (Fisher *et al*., 1978). It has been suggested that the development of the resistant population in a contaminated soil can result from vertical gene transfer or horizontal gene transfer, including transposons and broad host range plasmids and selection pressures on spontaneous mutants due to the presence of xenobiotics (Bogdanova *et.al.,* 1998). It has also been reported that pesticide-degrading plasmids are widespread in soil microbial populations. However, further detailed studies are required on the sorption behaviour of the fungicide in the soil before predicting its behaviour in the environment

CONCLUSION

The present study revealed that the *Stenotrophomonas maltophilia* is ubiquitously present in soil and can be exploited for Hexaconazole degradation. This study is beneficial as by having a detailed knowledge about the pesticide degrading bacteria because of the vast accumulation of xenobiotic on the earth and its non consumption directly by the plants and other organism. Such identification and characterization of degrading bacterial species is one of the very good strategies for environmental cleanup. Microbial biodegradation is a major process leading to transformation of organic chemicals in soil, and hence bioremediation is viewed as the most attractive approach to the ultimate destruction of exogenous agricultural chemicals added to cultivated soils. Recombinant DNA technology is a relatively new and fast growing area where the bacterial plasmids are being relatively used as cloning vehicles, because of its small size and relaxed mode of replication, resulting in multiple copies in a cell. Such plasmids increase the biochemical versatility of the host bacterium, extending the range of complex organic compounds used as sole sources of carbon and energy. In bacteria, these determinants are often found on plasmids, which have facilitated their study at the molecular level.

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