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Degradation of Lindane by Sludge Enriched on Mixed Commercial Formulations of Organophosphate and Pyrethroid Pesticides

Satish Kumar, Rajkumar Bidlan* and Jai Gopal Sharma

Department of Biotechnology, Delhi Technological University, Delhi- 110042, India
*Corresponding author

A B S T R A C T

Lindane ($\gamma-$Hexachlorocyclohexane) is detected in the environment even after three decades of its cessation of use. It is also found in milk, blood and food samples indicating the vastness of biomagnification. Organophosphates and pyrethroids are the other classes of pesticides being used frequently nowadays leading to further risk enhancement. Microbial degradation is the cost effective and safe strategy that can be developed and adapted for improving the environmental health by remediating lindane contamination. We enriched a bacterial population consisting of morphologically 47 distinct bacteria using commercial formulations of organophosphates and pyrethroids mixture. This consortium showed 55.6% - 90.45 % degradation of 5-30ppm lindane by 6 days. The temperature and pH optima were found to be 30°C and 6 respectively. When this consortium was induced with lindane in broth only four cultures survived while 24 isolates showed the ability to clear lindane film on a nutrient agar plate. This is the first report with a microbial population enriched completely on mixtures of commercial formulations of organophosphate and pyrethroid classes of pesticides and used for degrading a pure isomer of an organochlorine pesticide.

Introduction

Among all classes of synthetic pesticides, organochlorines have found indiscriminate applications in the last century. DDT (dichlorodiphenyltrichloroethane) and HCH (hexachlorocyclohexane) were the major OCP (organochlorine pesticides) used by many developed and developing countries. Lindane (γ- HCH) and DDT were detected in the water, sediments and biota of certain provinces in China even after 30 years of their ban (Grung *et al*., 2015). According to an estimate by Weber *et al*. (2008), approximately 6 million tonnes of

HCH isomers have been dumped into the environment. Vankarand Sahu (2011) have detected the average lindane levels of 10.03 ppm in a study conducted near the HCH dumping site by an HCH manufacturing unit in the Lucknow district (Uttar Pradesh state) of India. Lindane has been a causative agent to many health problems including respiratory disorders (Simonich and Hites, 1995; ATSDR, 2005; Ye *et al*., 2013). GABA neurotransmitter is influenced by lindane, affecting the vital organs like liver, kidney and the nervous system (Agrawal

and Shahi, 2015). Reduction ingermination of certain food crops in thepresence of HCH was observed by Bidlan *et al*., (2004). Lindane was also found in coffee samples of Romania (Stanciu *et al*., 2008). Nitrifying and denitrifying bacteria are also negatively affected by the presence of lindane (Tolido *et al*., 1993; Saez *et al*., 2006). Severe toxicity of lindane in fishes and gilthead seabream was demonstrated by Johnson and Finley (1980) and Oliva *et al*. (2008) respectively. Reports in the year 2000 (Cerkvenik *et al*.; Noren and Meironyte) showed the presence of organochlorine pesticides in cow and human milk from Slovenia and Sweden respectively. Concentrations exceeding the WHO/FDA limits of lindane have been detected in at least 50% of the of buffalo milk samples in national capital regions of India (Aslam *et al*., 2013). Similarly, milk samples from Lucknow (Uttar Pradesh state of India) were shown to have more than the maximum residue limit (MRL) of HCH (Nigam and Siddiqui, 2001); an increase by three times from the earlier studies by Saxena and Siddiqui (1982) indicating the increasing levels of HCH in the environment with time due to its liberal applications. According to a report from Centre for Science and Environment (CSE, India) 100% samples of blood from villages of Punjab state of India harboured lindane (Mathur *et al*., 2005). The use of pesticides seems to be unavoidable in the present scenario where new and resistant insect population is developing leading to heavy crop and economic losses.

The new generation of pesticides is also being brought into the market to combat this problem. Overall, the crops and health in all the biotic spheres areaffected negatively by the use of synthetic pesticides. Thus, it becomes imperative to develop strategies that can take care of these unprecedented levels of pesticides in our environment and help keep the nature clean and green. Here we report enriching a bacterial population that is resistant to a mixture of commercial formulations of non-organochlorine classes of pesticides and its efficacy towards lindane mineralisation.

Materials and Methods

Chemicals

Lindane (97%) was procured from Sigma Aldrich Co. (USA). Chlorpyriphos, propenofos cypermethrin, deltamethrin-triazophos and lambda-cyhalothrin were procured from Hindustan Pulverising Mills (HPM) Chemicals & Fertilizers Ltd. (India). Dichlorvos and cypermethrin were purchased from insecticide India Ltd (India) and Bharat Insecticides Limited (India) respectively. o-tolidine was purchased from Thomas Baker. All other chemicals were of analytical grade and purchased from standard companies.

Minimal Medium

The minimal medium was prepared according to Bidlan and Manonmani (2002).

Sample Collection and Preparation

Sewage and sludge were collected from surrounding areas of Delhi Technological University, Delhi, India (77.217 E long./ 28.667 N lat.), considering the fact that the wastes/effluents are dumped into these water bodies by almost every sector. The samples were maintained cold (4 ^{o}C) until further processing. Samples were mixed thoroughly just before use and filtered through Whatman No.1 filter paper. The filtrate was used as the source of native microbial population for the enrichment of pesticide tolerant microbes.

Enrichment of Consortium

To 20 ml sewage filtrate 80 ml distilled water and 0.5 g peptone was added. After shaking (120 rpm) for 24 h at room temperature (RT, 25- 30 ^{o}C), 2 ppm (2 mgL^{-1}) of each pesticide's EC was added and kept for shaking for 1 week. The cells were harvested and placed into a fresh 0.2% peptone with 2 ppm pesticide formulation mixture and kept shaking at RT. This process continued for a month with a gradual reduction in the supplied peptone concentration to 0.05%. After a month's treatment under these conditions, the pesticide concentrations were increased gradually up to 500 ppm of each of the commercial formulations. This process took 9 months. The viability of the microbes was regularly checked during the complete course of the process.

Inoculum for Degradation Studies

The consortium enriched by gradually increasing concentrations of commercial formulations of organophosphate and pyrethroid pesticides with naturally established ratio (after 9 months), was inoculated into nutrient broth (NB) and incubated for 24 h at RT. 5 ppm of lindane (as Dimethyl formamide solution, DMF) was added to the growing culture and was induced for 72 h at RT. This was harvested by centrifugation, washed twice in minimal medium and used as inoculum for degradation studies.

Effect of Temperature on the Degradation of Lindane

The induced consortium was inoculated to 20ppm lindane in minimal medium (in triplicates) maintained and incubated at temperatures 10°C through 50°C for 0, 3 and 6 days. The growth and degradation of the added 20ppm lindane were analysed for optimum temperature.

Effect of pH on Degradation of Lindane

0.1M phosphate buffers with pH 5, 6, 7 and 8 were prepared. 5 ml of each buffer was placed separately in triplicate test tubes and 10 ppm lindane (as DMF solution) was added. The induced consortium was inoculated to these tubes and incubated at RT. Controls for each pH and sampling periods were also placed without inoculum. The residual lindane and the growth were analysed.

Screening for Lindane-degrading Strains on Solid Medium

The pure cultures were isolated from the 9-month enriched consortium by repeated streaking on nutrient agar plates. Each culture was streaked for dense growth onto a fresh nutrient agar and allowed to grow for 24h. The plates were then sprayed with 0.5% lindane (in acetone) under aseptic conditions; acetone was allowed to evaporate leaving a thin lindane film behind. The plates were incubated at RT for another 48 h and observed for the zone of clearance in the lindane film dueto degradation by respective cultures.

Establishment of Cultures in Broth

The pure cultures from the original (9-month enriched) consortium were remixed in equal proportions of OD$_{600}$ and allowed to grow at RT in 1/50 nutrient broth for 72 h so that the natural proportion is established amongst the populations. At the end of 72 h of growth, 5 ppm of lindane (as DMF solution) was added to the culture. This was further incubated at RT for 72 h. The process was repeated for 3 cycles. The culture was streaked on nutrient agar plates for growth for 24-48 h to obtain the finally enriched lindane tolerant bacteria.

Analytical

Determination of Growth

One ml of sample was taken for spectrophotometry (Perkin-Elmer UV-Vis-NIR lambda950) and read at 600 nm against minimal media blank.

Extraction of Lindane

The samples were first acidified by adding 2-3 drops of concentrated HCl immediately after the sampling. The acidified sample was transferred into a glass separating funnel and extracted twice with equal volume of dichloromethane. The organic layer, carrying lindane, was passed through anhydrous sodium sulphate after standing for 5 minutes to separate the two layers. The organic layers for each sample were pooled and allowed to evaporate at RT followed by transferring to a microcentrifuge tube using acetone. The acetone was allowed to evaporate. The residual lindane was re-dissolved in a known volume of acetone for analysis by chromatography.

Thin Layer Chromatography

The residual lindane was estimated by using thin layer chromatography on silica gel G coated plates. The 20 x 20 cm^2 plates were prepared by spreading a uniform layer of 0.25 mm thickness silica gel G using a spreader. The plates were allowed to dry at RT and activated at 105 ^{o}C for one hour. A predetermined volume of the extracted lindane (in acetone) was spotted, developed in cyclohexane: n-hexane (4:1) solvent system as mobile phase till the solvent front reached 2/3 height of the plate. The plates were removed and the solvent was allowed to evaporate at RT. Lindane spots were detected by spraying 2% o-tolidine (in acetone) and exposing the plates to bright sunlight. The spots, peacock- green in colour, were delineated using a needle and the intensity observed and the spot area calculated. The square root of the area under each spot was linearly related to the logarithm of lindane concentration. The known amounts of lindane were spotted as standards for generating the reference curve.

Gas Chromatography

The residual lindane was confirmed and quantified by injecting known volume of appropriately diluted sample- extract dissolved in acetone into the gas chromatograph (Fison 1000 series) equipped with ^{63}Ni electron capture detector and SS column (200 cm x 2 mm) packed with 1.5% OV-17 plus 1.95 QF-1 on chromosorb- W, 80-100 mesh. The column, injector and detector were maintained at 230, 230 and 320 ^{o}C respectively with a flow rate of carrier gas nitrogen at 50 ml min^{-1}. Under these conditions, the retention time of lindane was 3.99 min. The detection limit of lindane by GC is 2 pg and above under the given set of conditions.

Results and Discussion

Enriched Population

At the end of 9 months of enrichment, morphologically 47 distinct bacteria were obtained on nutrient agar plates. These were purified by repeated streaking and maintained on nutrient agar with 5 ppm pesticide- mixture. All the 47 isolates were again mixed in equal quantities to reconstitute the consortium (undefined) and used for screening.

Degradation of Lindane by the Original Consortium

The consortium showed 55.61% - 90.35% degradation of lindane (Fig.1). The initial concentration of 5 ppm lindane was reduced

to 9.65% at the end of 144 h of incubation while initially added higher concentrations of 10, 15, 20, 25 and 30 ppm were reduced to 10.65, 29.21, 29.94, 39.25 and 44.39% during the same time. The corresponding growth of the microbial consortium is shown in Fig. 2(a &b). It was observed that the growth of the consortium increased drastically with increasing concentrations of lindane. The six- fold increase in growth on 30ppm lindane by 18 h is a reflection of utilisation of lindane as acarbon source by the members of the consortium (Fig. 2b).

It was observed that the growth followed a zig-zag pattern with the initial increase and then decrease and again an increase. At the end of 144 h, the final growth was approximately 2 ½ - 3 ½ folds with respect to the initial inoculum (Fig. 2a).

There appeared to be a direct correlation between the concentration and the time for degrading the initially supplied lindane. The other common trend observed was that when growth reduced, the degradation also diminished in that phase. This was an indication of the direct relationship between consortium growth and lindane degradation. Similar observations were made by Jilani (2013).

Lindane Degradation at Different Temperatures

The initially added 20 ppm of lindane was degraded by 13.89% at both 10 ^{o}C and 20 ^{o}C while 23.6% and 36.2% degradation was observed at 40 ^{o}C and 50 ^{o}C respectively (Fig. 3a).

Maximum degradation of 64.9% was seen at 30 ^{o}C (Fig. 3a). The corresponding growth is shown in Fig. 3b. Overall growth was observed only at 30 ^{o}C with 6.67% increase and at 40 ^{o}C with 34.6% increase in 6 days

of incubation. The added inoculum decreased at other temperatures. Even though the growth was better at 40 ^{o}C than 30 ^{o}C, degradation was better in the latter case. The reason may be the temperature optima for catabolic enzymes is in favour of the 30 ^{o}C. With 46% reduction in the inoculum at 50 ^{o}C, degradation was more effective than 40°C. This may be because of the higher inoculum size provided to 50°C and also certain catabolic enzymes (from certain member(s) of the consortium) might have higher temperature optima.

The consortium comprises of different cultures that may have different behaviour at different temperatures andpH. In an earlier study, Bidlan and Manonmani (2002) have earlier detected the deradation of DDT from 4°C to 50°C but the most efficient degradation was shown to be at 30°C while the efficient growth was observed at 37 ^{o}C.

Lindane Degradation at Different pH

The consortium could degrade the initially provided 10 ppm lindane by 13.9%, 18.9%, 23.6% and 50.5% at pH 5, pH 6, pH 7 and pH 8 respectively by the end of 144 h (6 days) of incubation (Fig. 4a). The corresponding growth pattern is shown in Fig. 4b.

It was observed that the growth increased by 42.86%, 52.17%, 39.13% and 86.96% in the first 3 days of incubation at pH 5, pH 6, pH 7 and pH 8 respectively that reduced to 14.29% for pH 5 and to 26.09% for other 3 pH respectively by the end of 6th day of incubation with respect to the initial inoculum level. The maximum growth increment in the first 3 days was at pH 8 followed by pH 6, pH 5 and pH 7 respectively while the reduction in next 3 days was least at pH 7 followed by pH 6, pH 5 and pH 8 with 33.33%, 50%, 66.67% and

70% reduction respectively. The degradation of lindane was more effective with an increase in pH indicating a direct correlation between studied pH levels and lindane degradation.

Even though the growth in pH 8 decreased maximum in last three days, yet the degradation was more efficient suggestive towards the secretory enzymes/enzyme systems involved in lindane degradation (this is our hypothesis and need further studies to prove; though it is believed that the degradative enzymes are non-secretory through experiments with Pseudomonas paucimobilis).

Bidlan and Manonmani (2002) and Bidlan (2003) have earlier shown that the pH 7- 7.5 was more favourable for bioremediation of DDT by *Serratia marcescens* DT-1P, *Pseudomonas aeruginosa* DT- ct1 and *Pseudomonas aeruginosa* DT- ct2. Sreedharan *et al*. (1999) demonstrated the degradation of HCH in acidic soils with pH above 3 using Pseudomonas paucimobilis. More growth during the first 3 days in pH 8 in our case, could have enhanced the enzyme production that continued to act during the subsequent three days of incubation, thereby degrading almost 87.29% of lindane as compared to first three days. At pH 6 the degradation in last three days was 225.32% as compared to the first three days. Since the growth in pH 6 was second to that of pH 8, the greater degradation percentage in thesecond half as compared to the first half might be due to the pH optima for the catabolic enzymes. The culture can be grown at pH 8 initially and then the pH 6 can be provided for efficient degradation. The pH 7 showed the least increase in biomass in the first half but it also showed the least decrease in the biomass in thesecond half; thereby giving the consortium optimum condition to

degrade 69.89% lindane in last three days as compared to the first three days. Yet, the most efficient degradation among different pH provided was at pH 8. Earlier, Bidlan and Manonmani (2002) found that the degradation and growth reduced at pH greater than 7.5.

Screening of the Individual Isolates for Lindane Degradation on Agar

Cultures 3, 5, 6, 7, 9, 10, 11, 14, 15, 18, 19, 20, 22, 23, 26, 28, 29, 30, 33, 35, 36, 38, 44 and 46 showed clearance of lindane film (Fig. 5). This was an indication that the cultures have the potential for degrading lindane.

Screening of the Individual Isolates for Lindane Degradation in Broth

It was observed that the lindane- induced consortium had dominatingly 4 distinct cultures. These cultures were purified, designated as LR1, LR2, LR3 and LR4 respectively (Fig. 6) and maintained on minimal agar supplemented with 1/50 NB and 5 ppm lindane for future studies later.

Most reports show that the enrichment is done with the same (parent) compound (Manonmani *et al*., 2000; Bidlan, 2003; Bidlan *et al*., 2004; Nagpal and Paknikar, 2006; Murthy and Manonmani, 2007; Deepthi *et al*., 2007; Pannu and Kumar, 2014) or the analogues of the compound (Focht and Alexander, 1970; Bartha, 1990) that needs to be degraded by the microbes. In contrast to the basic practice, our approach was to adapt a technique that could be beneficial in the present and future scenario, keeping in view that most parts of the world are now applying the organophosphates and pyrethroids instead of organochlorine pesticides.

Amyotrophic Lateral Sclerosis (ALS), a progressive fatal neurodegenerative human disease has been associated with pesticide exposure especially with organochlorines, pyrethroids, herbicides and fumigants (Kamel *et al*., 2012) and organophosphates (Chen, 2012). Slotkin and Seidler (2009) have demonstrated the neurological and neurotoxic effects of organophosphates while David *et al*. (2014) discussed the teratogenic effect of paraoxon, an organophosphate, on neurodifferentiation.We wish to establish a population tolerant to the existing concentrations of non- organochlorine pesticides in the environment and probably still higher concentrations in future. Being tolerant to these other classes of pesticides will help the application of our cultures as they would stay active and remediate the organochlorine load already present in nature. Apart from this, the same culture might be able to remediate the loads from other classes of pesticides.

Grung *et al*. (2015) stressed the need for research on organophosphates since they are the dominating group being used at present. Viel *et al*. (2015) discussed the cognitive developmental disabilities in post- natal stages of children due to the exposure of pyrethroids leading to an urgent realisation of considering the remediation for this pesticide class as well. Our cultures from the consortium may act upon these pesticides as they were enriched by the mixture of organophosphates and pyrethroids. The probability of the application of our consortium towards simultaneous degradation of different classes of pesticides cannot be ruled out. Selvi *et al*. (2013) worked with DDT- degrading consortium to study the degradation of the pesticide isoprothiolane. Pino and Penuela (2011) developed a microbial consortium with 12 different bacterial strains through enrichment technique that could degrade

150 ppm each of chlorpyrifos and methyl-parathion separately as well as simultaneously. Liu *et al*. (2009) engineered strain LZ1 of Stenotrophomonas species that could produce the enzyme organophosphorus hydrolase (OPH). This enzyme was capable of degrading p-nitrophenol substituted organophosphates and their products. The three organophosphates paraoxon, parathion and methylparathion degradation was enhanced in the presence of 4-chlorophenol. Yang *et al*. (2012) could co-express the lin A_{and}mpdgenes in a cloned E. coli for simultaneous degradation of organochlorine (lindane) and organophosphate (methyl parathion). This clone is good to work in a bioreactor as it also expresses the green fluorescent protein for detection. Our consortium on the other hand, with longer acclimatisation, may work in-situ. The need of acclimatisation (pre-exposure or induction) in bioremediation was earlier emphasised by many researchers in the past as well (Bidlan and Manonmani, 2002; Bidlan, 2003; Jilani, 2013). Sonkong *et al*. (2008) also demonstrated the effectiveness of 72 h induction of inoculum to DDT in the presence of glucose and yeast extract for enhancing the degradation of DDT. Successive exposure of culture to lindane improved its ability to degrade the pesticide (Wada *et al*., 1989; Bhuyan *et al*., 1992) while the same with DDT did not yield any improvement (Bidlan and Manonmani, 2002).

Microorganisms transform the complex organic compounds to CO_2 or other simple organic compounds through their metabolic enzymes. This reducing equivalents from the oxidation are assimilated and results in the growth of organisms that work out the degradation (Latha, 2012). Our results also reflect the similar view along with the other hypothesis proposed here by us that the degradation during the decline phase of

growth may be due to the enzyme/ enzyme systems already synthesised by the microbial cells during their active growth phase (need further studies to prove). Baczynski *et al.* (2010) noted the initial increase in growth with substantial decrease and then the biomass remaining constant until the end of the studies. The zig- zag pattern of growth as in the present study was also observed by Sander *et al.* (1991), Bidlan and Manonmani (2002), Sonkong*et al.* (2008) and Jilani (2013). Jilani (2013) discussed the decline in the viable count due to non- acclimation of the culture to the pesticides while we hypothesise it differently here; further studies might establish the fact.

Fig.1 Degradation of Lindane by the Consortium Enriched on Mixture of Organophosphate and Pyrethroid Pesticide (Commercial Formulation)
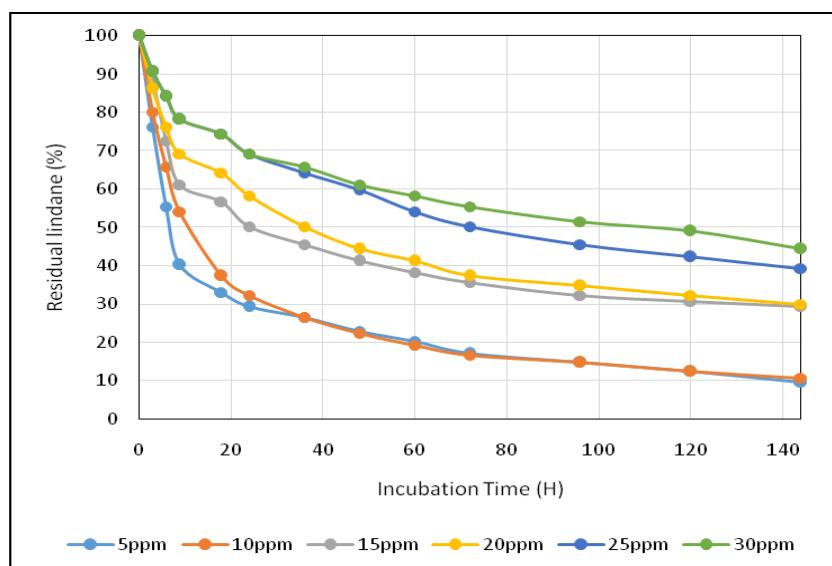
Fig.2a Growth of the Consortium on Different Concentrations of Lindane. The Consortium is Enriched on Mixture of Organophosphate and Pyrethroid Pesticide (Commercial Formulation)
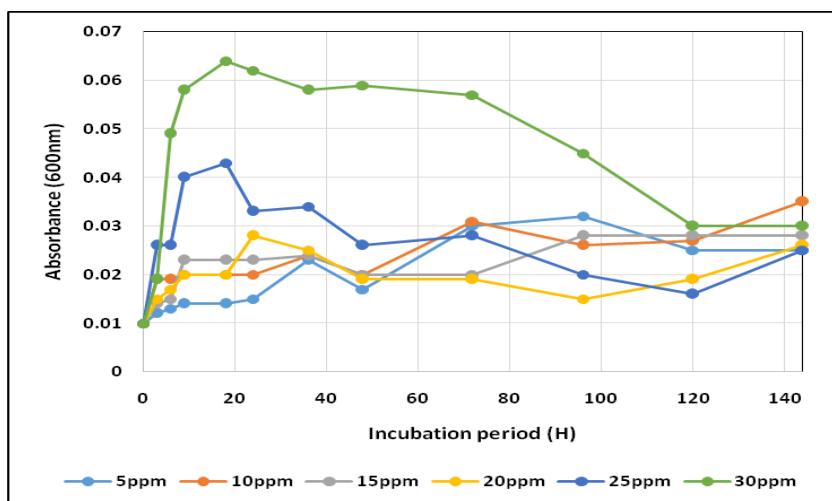
Fig.2b Growth of the Consortium on Different Concentrations of
Lindane During the First 24 h
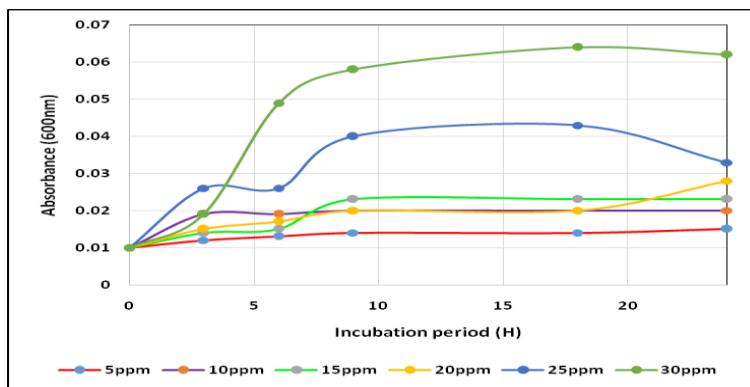
Fig.3a Degradation of Lindane by the Consortium. The Consortium was Inoculated to 20ppm
Lindane in MM and Samples were Drawn at 0 Days, 3 Days and 6Days for Analysis
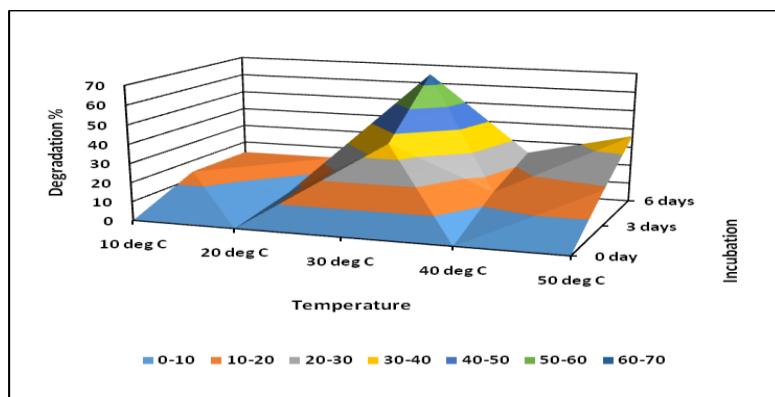

Fig.3b Growth of the Consortium at Different Temperatures. The Consortium was Inoculated
to 20ppm Lindane in MM and Samples were Drawn at 0 Days, 3 Days and 6Days for
Analysis
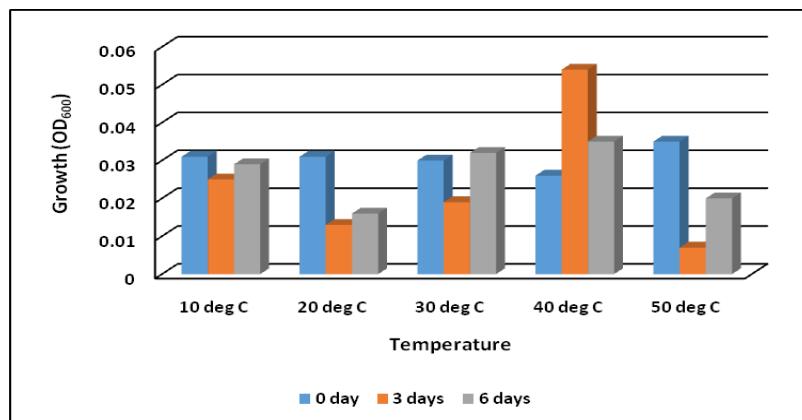
Fig.4a Effect of pH on Lindane Degradation by the Consortium. The Consortium was Inoculated to 10ppm Lindane in MM and Samples were Drawn at 0 Days, 3 Days and 6Days for Analysis
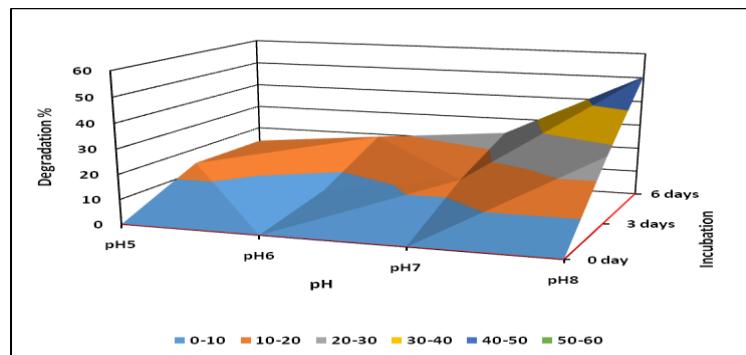
Fig.4b Effect of ph on Consortium (Biomass). the consortium was Inoculated to 10ppm Lindane in MM and Samples were Drawn at 0 Days, 3 Days and 6Days for Analysis
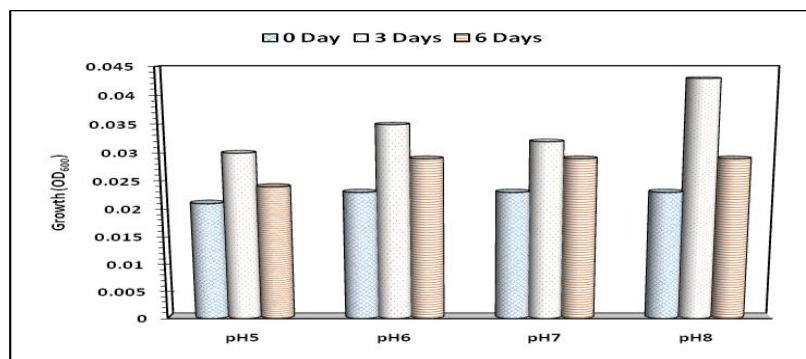

Fig.5 Individual Strains were Sprayed with 0.5% Lindane Solution to form a Thin Film and Incubated at R.T. The Cultures that have the Potential for Degrading Lindane Cleared the Film
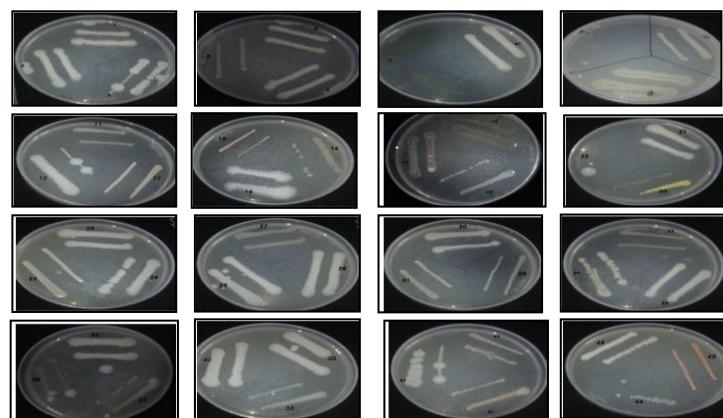
Fig.6 Individual Strains that Ultimately Survived the Lindane Enrichment
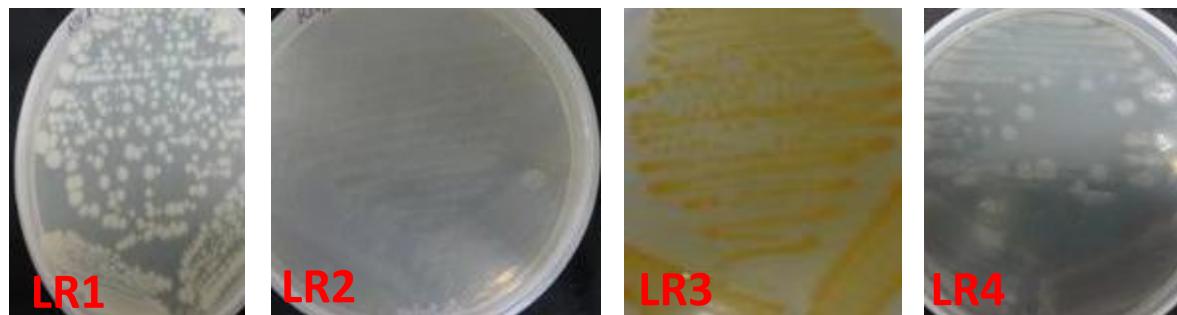
Acclimation of consortium for DDT degradation by Bidlan and Manonmani (2002) also resulted in the ultimate survival of four strains comprising *Serratia marcescens* DT- 1P and other three Pseudomonas strains. Pannu and Kumar (2014) isolated 78 strains out of which only 9 strains could clear the lindane film after 7 days of incubation and only 3 strains RP-1, RP-3and RP-9 were able to withstand 100 ppm of this insecticide. The other strains were able to tolerate lindane concentrations from 20- 60 ppm (parts per million= 1×10^{-6}). Similar observations with limiting cadmium were described by Kumar *et al*.(2010). The cultures in pure form were able to degrade lindane in agar medium while in the broth where the cultures were in mixed form, the total number of surviving/ actively growing cultures were observed to be only four (Fig. 6). This might be due to the interaction between the various types of bacteria present. The two cultures viz. RL2 and RL4 were dominating and appeared indiscriminately, overcasting the other members of the consortium. This might be due to the antibiotic or inhibitory factors produced by the two cultures. The other two cultures (RL1 &RL3) were able to resist these factors along with the lindane; hence survived.

The degradations of pesticides have been achieved in many works in the past 50- 60 years. The time required and the percentage of degradation varied from case to case. The microbe used and the type of pesticide under study play a vital role than the other parameters like pH and temperature. Many times the significant parameter described is the inoculum size (Bidlan and Manonmani, 2002; Selvi *et al*., 2013). There might also be the effect of a threshold level of the initial pesticide concentration necessary for the degradation below which the culture induction is not possible. More investigations may further support the findings.

In conclusion, this is the first report of its kind with a microbial population enriched completely on mixtures of commercial formulations of organophosphate and pyrethroid classes of pesticides used for degrading a pure isomer of an organochlorine pesticide. The fact that our consortium could degrade substantially the initially supplied lindane concentrations from 5 ppm through 30 ppm in a short period of 6 days is a positive sign to apply it for remediation of contaminated water bodies; of course, after further vigorous training (acclimation) of the consortium with lindane in presence of the organophosphates and pyrethroids. The future environmental problem may arise through the indiscriminate use of organophosphates and pyrethroid pesticides.

For such conditions prevailing in future, our consortium and its application can become the master stroke. The avenues are still open for a better combinatorial bioremediation strategy to save our biosphere in a healthy way for the future generations.

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