Study of Pretilachlor Degradation by Microbes Isolated From Rice Field of Jabalpur Region

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Abstract- ***The proposed study was carried out to assess the fate of pretilachlor under the influence of microbes isolated from rice field of soil and water. Biodegradation, both in soil and in the media by microbes indicated that the appropriate consortium of can remove pretilachlor herbicide from soil and water. Best degradation of pretilachlor was done by Aspergillus flavus and Klebsiella species. The degradation product of pretilachlor formed by Klebsiella species in media metabolite – I, 2', 6'-diethyl-N-(2-hydroxyethyl) aniline, metabolite-II 2', 6'-dimethyl-N (propoxyethyl) hydroxyl annelid, metabolite-III 2-chloro-2', 6'-diethyl acetanilide, metabolite-IV chloro-N-methyl acetamide, in soil metabolite-V 2', 6'-diethyl-N- (propoxyethyl) aniline and in water two other metabolite-VI 2-Propoxy-N-(propoxyethyl) acetamide and metabolite-VII 2-hydroxypropoxy-N-(propoxyethyl) acetamide formed by same species. There are two substitutions in the aniline-N of pretilachlor- one is chloroacetyl group and the other is propoxyethyl group formed by Aspergillus flavus.***

Index terms: Pretilachlor, ***Degradation, Klebsiella, Aspergillus Niger.***

I. INTRODUCTION

Previous study support that the herbicides belong to a group of chemicals renowned as pesticides, which prevent, destroy or mitigate any weed. These are any chemical substance that is used to specifically kill harmful plants. Other familiar pesticides are insecticides and fungicides. These herbicides are designed to kill or control specific plants which were harmful to crop as well as human beings, so a great deal is known about the acute biological effects of these chemicals on their target organisms. They are control or kill plants through a variety of mechanisms, including the enzyme function, root growth, interference with the synthesis of pigments, proteins or DNA, destruction of cell membranes, or the promotion of uncontrolled growth, inhibition of biological processes such as photosynthesis, mitosis, cell division, enzyme function, root growth, interference with the synthesis of pigments, proteins or DNA, the promotion of uncontrolled growth or destruction of cell membranes reported by William *et al.* [1].

Herbicides reaching the soil in significant quantities have a direct effect on soil microbiological aspects, which influence the plant growth. The most important effects caused by herbicide are inhibition of N_2 fixing soil microorganisms such as *Rhizobium*, *Azotobacter*, *Azospirillum* species. Many herbicides reported for the suppression of nitrifying bacteria, *Nitrosomonas* and *Nitrobacter* reported by various scientists. From the agricultural point of view, this longer persistence of herbicides leading to accumulation of residues in soil may result into the increased absorption of such toxic chemicals by plants, and these plant products consumed by human beings as well as other mammals may prove harmful.

Most herbicides are poisons; considerable knowledge has also been developed regarding the acute effects of these compounds in humans. Rather, more knowledge is essential to know the unseen sub lethal effects on non target organisms due to chronic exposure to pesticide compounds. Some effects of chronic pesticide exposure in human being and wildlife include endocrine disruption [2], cancer [3], lung cancer [4], immune system suppression, Parkinson's disease [5] and fetal death [6]. These are only possible when our food chain, potable water and air are contaminated heavily with herbicide. To avoid this contamination it is essentially important to know the fate of herbicides in the environment, more specifically in the water.

In aquatic environment, herbicides mainly undergo biotic degradation by microbes. The breakdown of herbicides by a soil organism known as microbes accounts for a large portion of herbicide degradation in soil. Some micro-organisms are herbicide specific, and their community in the soil is related to the amount of herbicide available for their consumption. Repeated use of herbicide can lead to increased microbial population and as the shorted duration of effective weed control. The term 'biodegradation' or 'biotransformation' is a natural process in the environment by which any organic compound or naturally occurring xenobiotic is enzymatically degraded initially into smaller fragments and finally to its minerals. Enzymes, which act as biocatalysts for the degradation of organic substrates,

are released by living organisms including organism, viz. Bacteria, fungi and actinomycetes etc. While degrading the organic compounds by enzyme microbes extract energy from those compounds for their survival and proliferation [7].

The breaking down or transformation of a complex substrate into simpler products leading finally is to mineralization. Degradation is considered to be synonymous with mineralization, e.g. Thirum (fungicide) is degraded by a strain of *Pseudomonas* and the degradation products formed by these strains are dimethlamine, proteins, sulpholipaids [8]. It is also important to know the process of detoxification of these dangerous chemicals present in water and soil. In this view pretilachlor, a chloroacetanilide herbicide has been chosen for the study on its degradation by microorganism, i.e. biodegradation and its decontamination in water and soil by these microorganisms, i.e. bioremediation. Pretilachlor (2-chloro-2′, 6′-diethyl-*N*-(2-propoxyethyl) acetanilide) is a soil-applied herbicide selectively used in rice fields.

Microbes play very effective role in degrading the herbicide and also xenobitotic materials in the soil and applied for management pest and weeds in the agricultural crops. In this study rice (*Oryza sativa L*) has been selected for the study of the degradation of weed in the rice /paddy field. Rice (*Oryza sativa L.*) is a principal source of food for the world's population, with about two-thirds of total rice production grown in irrigated environments. It is a staple food, providing livelihood to more than 1.2 million farmers reported by Abeysekera *et al* [9]. It is commonly grown as direct-seeded rice, wet-seeded and dry-seeded. Pretilachlor disappears from the paddy water at a moderate rate with a half-life of 3.0 to 3.6 days [10]. But in general, the half-life of pretilachlor in soil is 20 to 50 days [11]. It, therefore, will get enough time to contaminate water bodies, either the ground water or the surface water. The fate of pretilachlor and its decontamination process in soil and water are yet not investigated extensively. In the present investigation, pretilachlor, a soil applied rice herbicide has been selected as test chemical. Pretilachlor degrading microbes were screened out from soil and water. The rate of degradation and mode of degradation of pretilachlor by those individual microbes and by their consortium was studied in media, soil and water.

II. MATERIALS AND METHODS

Chemicals-

For the present study pretilachlor herbicides used for weed control around rice fields of the DWSR (Directorate of Weed Science Research Institute) Maharajpur, Jabalpur, were selected. These herbicides composed from technical pretilachlor (90.5%) and analytical pretilachlor (99.9%) and obtained from Indofil Chemicals Company, Mumbai. The purity of this pretilachlor was checked by thin layer chromatography. Laboratory grade reagents and solvents were locally procured, purified and used for study. A.R. grade chemicals of Merck were used for present study and some chemicals were purchased from Hi-media, India.

Sample Collection-

For the present study various soil and water samples were collected from different rice fields in Jabalpur region, were selected having the history of pretilachlor application for consecutive five years. Soil and water samples were collected at different cropping periods in the middle of the growing season when rice was grown. The soil samples were collected from the root region of rice by digging 10-15 cm depth below the soil and some part of the adjacent soil is taken assuming nitrogen richness in the soil. Similarly water samples were collected around the root region of plant parts. Both samples were collected in a sterile glass container, sealed and carefully placed in plastic bags and brought to the laboratory.

Physico-chemical characteristics of soil-

Composite samples were taken randomly from different spots in 0-15 cm to record the initial physico-chemical characteristics of the experimental soil. The soil samples were air-dried, grounded up and sieved through a 2 mm sieve and subjected to tests on their properties according to Olsen *et al.* and Subbiah and Asija, [12], [13]. Physico-chemical characteristics of experimental soil are presented following:

Electrical conductivity	Carbon	Nitrogen	Phosphorus	Potassium
0.30 dsm^{-1}	0.60% was	140 kg ha^{-1}	41 kg ha^{-1}	150 kg ha^{-1}

Serial dilution method-

The serial dilution method used for the isolation and enumeration of fungi and bacteria [14].

Dilution of soil samples:

The soil sample was mixed thoroughly to make a composite soil. Then 10 gm of sub-soil sample diluted to 100 ml distilled water that considered 10^{-1} dilution factor. 1 ml of 10^{-1} dilution transferred to 9 ml sterilized water with the help of sterilized pipettes for making 10^{-2} dilution. In this way, a series of up to 10^{-5} dilution was prepared under aseptic condition for the isolation of bacteria and fungi. Screw cap test tubes and glass petri dishes were used for isolation and culturing of microorganisms. Sterility is the hallmarks of successful works in the

microbiological studies were also kept in mind throughout the study.

Dilution of water samples:

10 ml water sample take with the help of micropipette as stock sample and dissolved in 90 ml of distilled water that idealized 10^{-1} dilution factor. Then transferred of 1 ml of 10^{-1} dilution to 9 ml sterilized water with the help of a sterilized pipette to make 10^{-2} dilution. In this way, a series of up to 10^{-5} dilution was prepared

Sample inoculation-

Fungi:

1 ml of diluted soil and water sample (dilution 10^{-4}) was inoculated on to the Rose Bengal media for fungal growth and to stop the growth of another organism (bacteria) add 40 mg Streptomycin powder (Lupin, India). The plates were wrapped and kept in an incubator for 48 hours at 28°C. After 48 hours the plates were observed for the fungal growth.

Bacteria:

For the isolation of bacteria from the samples 10^{-2} dilution was taken and used for plating on Nutrient Agar Media (NAM) (Hi Media Laboratories, India). All the plates were allowed to grow at 37°C in a bacteriological incubator for at least 24-48 hours for bacterial growth. The microorganism's total count was obtained by multiplying the number of cells per plate by the dilution factor, which was the reciprocal of the dilution.

Isolation of pure culture-

After 24 – 48h incubation well-developed and separated colonies were observed on the surface of a Rose Bengal medium (for fungi) and Nutrient Agar Medium plate (for bacteria). The culture of each colony was picked up with a sterile needle and transferred separately in PDA (fungi) and NAM (bacteria) slants. Each of these new slant cultures represents the growth of a single species. The colonies, which are different in appearances and characters, were picked and purified.

Preparation for microscopic examination-

The identification of bacteria and fungi was done on the bases of their morphological and physiological features. For the identification of bacteria, Gram staining method and lacto phenol cotton blue were used for the study of the fungi.

Identification of fungal strains:

The culture characteristic of each colony such as colour, texture, appearance was recorded. For further identification, fungal cultures were stained and viewed under microscope. For this, fungal culture stained with lacto phenol cotton blue describe in my previous paper. The microscopic characters of the culture, such as shape, size, colour, pattern and arrangement of mycelium, conidia, conidial heads, conidiophores, spores, sporangia was recorded. Identification was done with the help of available literature, including research papers, monographs, book etc. All the isolated fungus culture was identified up to genus or species level on the basis of their cultural and morphological characteristics observed under microscope and relevant literature. All the cultures of isolated fungal strains were maintained in PDA slants with streptomycin at 28°C during the study.

Identification of bacterial strains:

Different identification keys and protocols were used for bacteria identification. The bacteria were identified by morphological characters of the colony, Gram's staining and biochemical tests [14]. For bacterial staining a small portion of the bacterial culture was taken on slide with the help of sterile loop. The suspension was sufficiently diluted. A drop of the suspension was spread evenly covering an area of about 10-15 mm in diameter. The slide was air dry and the smear fixed then by rapidly passing on the flame and the slide was allowed to cool before staining. Different biochemical test, i.e. Indole, Methyl Red, Voges-proskaur, Citrate, Urease and Triple sugar Iron agar were used for the identification of bacteria [15]. After the isolation of bacteria all cultures maintained in pure culture. To allow continued growth and viability of microorganism all the pure cultures were transferred periodically into a fresh nutrient medium (sub-culturing). The transfer is always subject to an aseptic condition to avoid contamination [16].

Screening of Pretilachlor degrading microbes: The solution of pretilachlor was prepared in acetonitrile.

For Fungi:

For screening of pretilachlor degrading fungi 5 mg, 25 mg, 50 mg, 100 mg, 200 mg /L^{-1} pretilachlor was added in 250 ml conical flasks containing 200ml PDA broth. The pretilachlor was allowed to dissolve overnight on a shaker before inoculation of fungi. When pretilachlor was completely dissolved, isolated fungi inoculated into a flask and kept the dark at $25^0C \pm 2^0C$ for 60 days in a B.O.D. incubator.

For Bacteria:

For screening of pretilachlor degrading bacteria 5 mg, 25 mg, 50 mg, 100 mg, 200 mg /L^{-1} pretilachlor was added in 250 ml conical flasks containing 200ml NAM broth. The pretilachlor was allowed to dissolve overnight on a shaker before inoculation of bacteria. When pretilachlor was completely dissolved, isolated bacteria inoculated into the flask and kept the dark for 60 days.

Degraded products were extracted (for bacteria and fungi) by partitioning in dichloro methane (50 ml x 3) after 60 days of incubation. Then solvent was evaporated by water bath to obtain a crude mixture of products. A compound in the crude extract was separated by and characterized by spectroscopic technique [17].

Extraction and characterization of metabolites-

Degraded products were extracted from broth in a different time interval by partitioning in chloroform. The incubated soil was also extracted with ethyl acetate by continuous shaking for 4 hours and filtered. The solvent was then evaporated under low pressure in the rotary vacuum evaporator to obtain a crude mixture of products. The products were purified and characterized by the following chromatographic and spectroscopic techniques.

Chromatography -

Thin layer chromatography:

Silica gel based TLC plates were prepared by spreading a slurry of silica gel G containing 10 % binder (gypsum) in water on 6 cm X 20 cm glass plates, uniformly maintaining a thickness of 0.75 mm using a TLC applicator. Prepared plates were air dried first and then activated at 120 ^{0}C for 2 hours. The sample solutions were spotted on the TLC plates using capillary tubes. Plates were developed in suitable solvent systems, air dried and visualized by iodine vapour. The technical pretilachlor was chromatograph by TLC (solvent hexane: acetone, 3:2 v/v). The relative front (R_f) value of the active compound was measured by dividing the distance travelled by the compound with the distance travelled by the solvent. For the preparative TLC, 1mg of fluorescence indicator was added during the preparation of silica gel. Glass plates of 20cm X 20cm size were coated with this slurry. Spots were visualized under UV light, marked and scrapped. The scrapped silica gel was extracted with suitable solvent.

Liquid Chromatography-Mass Spectroscopy:

An API 3200 Qtrap mass spectrometer hyphenated to Shimadzu UFLC was used to mass characterization of degraded products. For each degraded product mass spectrometric analysis method was performed with electro-spray ionization (ESI) in positive (5500 eV) mode. The nebulizer gas and heater gases were adjusted at 30 psi and 55 psi, respectively. The ion source temperature was set at 500^{0}C. Each sample was injected by infusion technique at the rate of 10 µLs^{-1}.

III. RESULT

Isolation and Identification of microbes-

For the present study soil and water samples were collected from the different rice fields of the DWSR (Directorate of Weed Science Research Institute), Jabalpur. When the soil and water samples were cultured, two bacteria, namely *Pseudomonas* and *Klebsiella sp.* and eight fungal cultures namely *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum*, *Aspergillus* terreus, *Aspergillus* fumigatus, *Penicillium notatum*, *Trichoderma sp*. and *Curvularia* sp. were isolated from the rice field in the Jabalpur region.

Table 1: Degradation rate of pretilachlor screened by different microorganisms isolated from rice field.

Day after incubation	Remaining pretilachlor (%)			
	Pseudomonas sp.	*Klebsiella sp.*	*Aspergillus flavus*	*Aspergillus terreus*
0	100 ± 0.00*	100 ± 0.00	100 ± 0.00	100 ± 0.00
15	87.50 ± 5.53	63.93 ± 10.58	65.38 ± 15.95	92.12 ± 2.26
20	77.14 ± 4.06	45.90 ± 4.17	44.64 ± 6.11	81.93 ± 2.33
25	56.00 ± 8.07	39.81 ± 5.48	35.78 ± 2.95	54.46 ± 9.72
45	38.44 ± 2.25	34.38 ± 3.5	22.85 ± 4.69	23.70 ± 2.83

*Mean of three replications ± SD

The rate of degradation of pretilachlor by different microorganisms in soil and water- These isolated fungi and bacteria survive in 5mg to 200 mg pretilachlor. This result indicates their ability to degrade pretilachlor as a source of nutrients for their growth. The rates of degradation of pretilachlor were screened against two bacteria and two fungi in miniature and the best activity shown by *Klebsiella sp*. and *Aspergillus flavus* were shown in Table 1. The patterns of degradation in all the cases were similar to each other in earlier days and the extent of degradation after 45 days of incubation increased by fungi comparative bacteria. After 45 days of incubation, about 65% of the applied pretilachlor was metabolized by the bacteria and about 76% was metabolized by the fungi.

Degradation of pretilachlor by Klebsiella –

Klebsiella degraded pretilachlor by releasing extracellular enzymes, which act on it and convert it into simpler forms enabling the microorganism to derive energy from the herbicide for their growth and maintenance. These simpler forms of herbicide are usually called metabolites. Pattern of degradation postulated through periodical sampling that involved 0, 5, 10, 15, 20, 25, 30, 45 and 60 days of incubation of samples. The degradation of pretilachlor was monitored by TLC and the products were analyzed by LC-MS/MS and characterized by their mass ions and fragmentation patterns. The degradation pattern was observed in media, water and soil. Seven degradable products isolated during the study.

In the media: The media containing pretilachlor were incubated with *Klebsiella species* after 20 days of incubation, the media was extracted with ethyl acetate. The extract was then cleaned up and analyzed by LC-MS/MS. Four metabolites were isolated and characterized by their respective mass spectra (Fig. 1).

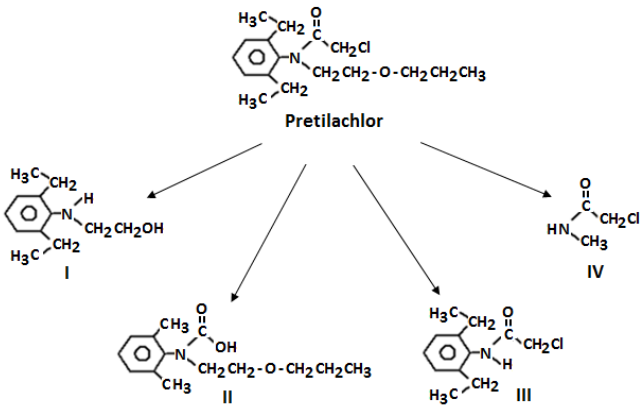

Fig. 1: Degradation of pretilachlor by *Klebsiella species* in media with the formation of four metabolites.

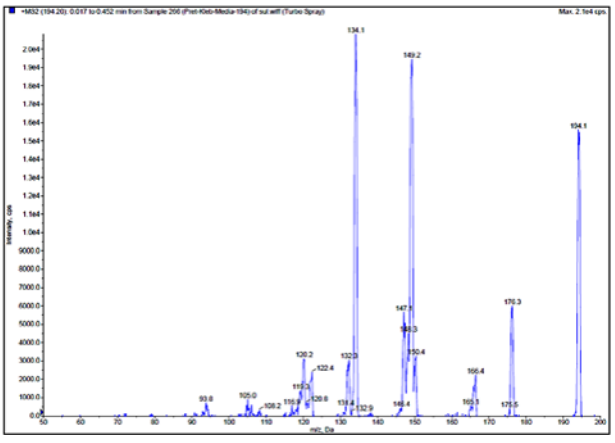

Fig. 2: Mass spectrum of 2', 6'-diethyl-*N*-(2-hydroxyethyl) aniline (I).

Metabolite-I: The mass spectrum (Fig.. 2) of this compound shows a strong peak at 148 indicating the presence of 2',6'-diethyl aniline, which is further confirmed by the fragment 2,6-diethyl phenyl (M/Z 133). The peaks at M/Z 162 and 176 indicate the attachment of one ethyl group (-CH_2-CH_2-). The molecular ion peak appears at M/Z 191, which is M-2 of the molecular mass 193. The loss of 17 amu confirms the presence of one hydroxyl group. Therefore, from this fragmentation pattern the structure of the compound is assigned as 2', 6'-diethyl-*N*-(2-hydroxyethyl) aniline.

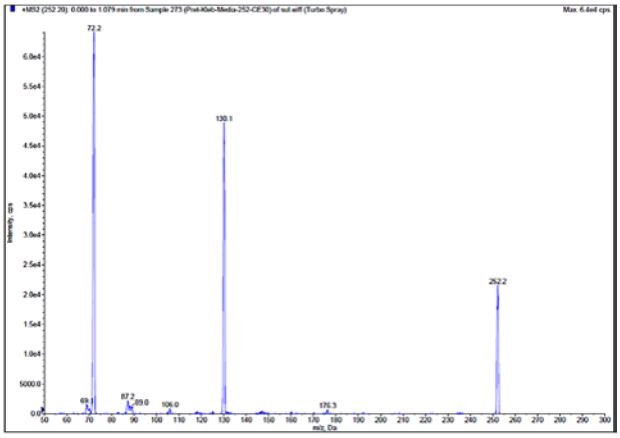

Fig. 3: Mass spectrum of 2',6'-dimethyl-*N*-(propoxyethyl) hydroxyl anilide (II).

Metabolite II: In the mass spectrum (Fig. 3) The peak at M/Z 252 is for the protonated molecular ion (MH^+), which on loosing consecutively, one –OH (17 amu) and one -C=O (28 amu) groups generated a fragment ion at M/Z 207 (Fig. 2). The presence of a peak at 106 and absence of a peak at 134 indicates the presence of diethyl moiety. The peak at M/Z 130 is probably of CH_3-$(CH_2)_2$-O-$(CH_2)_2$-N-CO group, which is further confirmed by the presence of CH_3-CH_2-N-CO group at M/Z 72. Computing these fragments the structure of the compound is assigned as 2', 6'-dimethyl-*N*-(propoxyethyl) hydroxyl anilide.

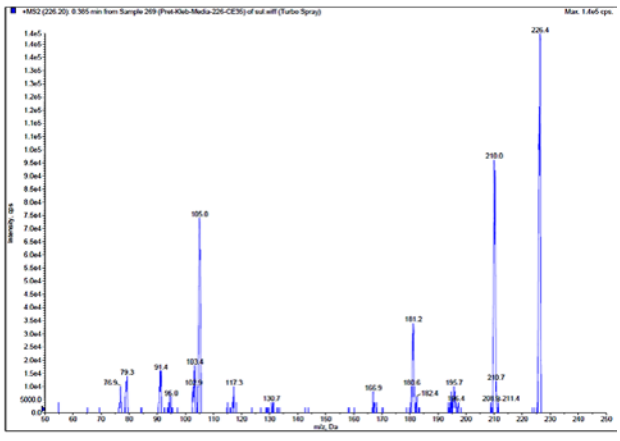

Fig. 4: Mass spectrum of 2-chloro-2',6'-diethyl acetanilide (III).

Metabolite III: A protonated molecular ion peak was observed at M/Z 226 in the mass spectrum (Fig. 4). Losing a $–CH_3$ group the molecule led to the formation of a fragment ion at M/ Z 210 amu. The fragment ion at M/Z 195 is due to the loss of one $–CH_2$-CH_3 group from the molecular ion. The peaks at M/Z 181 and 167 are due to the losses of a $–CH_3$ group and a $–CH_2$-CH_3 group from the fragment ion of M/ Z 195.These losses of two ethyl groups from the molecular ion indicate the presence of two ethyl groups in the compound. The peaks at M/z 79 and 91 indicate the presence of –N-CO-CH_2-Cl. Thus, after computing all these fragments the compound is assigned as 2-chloro-2', 6'-diethyl acetanilide.

Metabolite IV: The mass spectrum (Fig. 5) shows a strong peak at 108 as MH^+ peak. The dissociation of CH_2Cl from the molecule leads to the formation of a fragment (CH_3-NH-CO) at M/Z 58.A very small peak at M/Z 92 indicates the generation of demethylated fragment (NH-CO-CH_2Cl). From this fragmentation pattern (Fig. 4) the tentative structure of the degradation product is assigned as chloro-*N*-methyl acetamide.

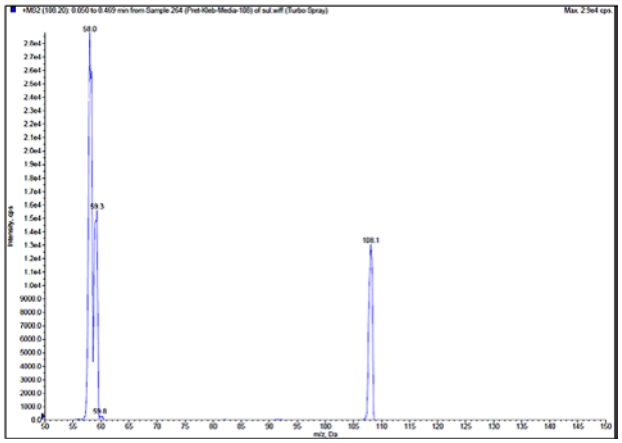

Fig. 5: Mass spectrum of chloro-*N*-methyl acetamide (IV).

In soil: Sterilized soil containing pretilachlor was incubated with *Klebsiella* sp. The incubated soil was extracted with ethyl acetate and cleaned up. The extract was then analyzed by LC-MS/MS. Five metabolites were isolated and characterized by their respective mass spectra (Fig. 6). Metabolites I to IV were also isolated in incubated media and characterized described above.

Metabolite V: The mass spectrum (Fig. 7) shows that fragments at M/Z 120, 134, 147 and 148, which indicate the presence of 2,6-diethyl aniline moiety in the structure. The peak at M/Z 176 is due to ethylene substituted aniline derivative, to which the addition of one –OH group generates the peak at M/Z 183. A propyl (-$CH_2CH_2CH_3$) group, when replaces the H of –OH in the fragment at M/Z 183 leads to the formation of a protonated molecular ion (MH^+) at 215. The compound is assigned as 2', 6'-diethyl-*N*-(propoxyethyl) aniline.

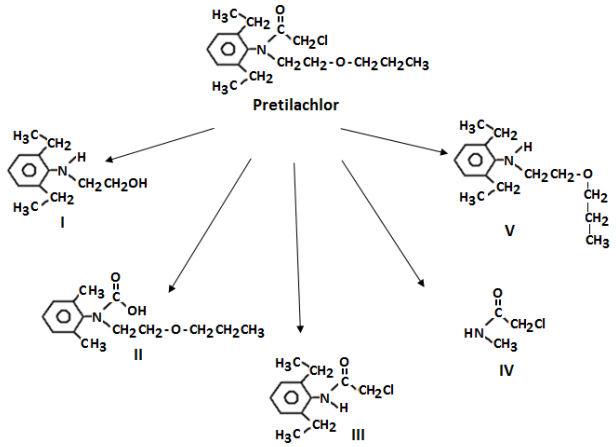

Fig. 6: Degradation of pretilachlor by *Klebsiella species* in soil with the formation of five metabolites.

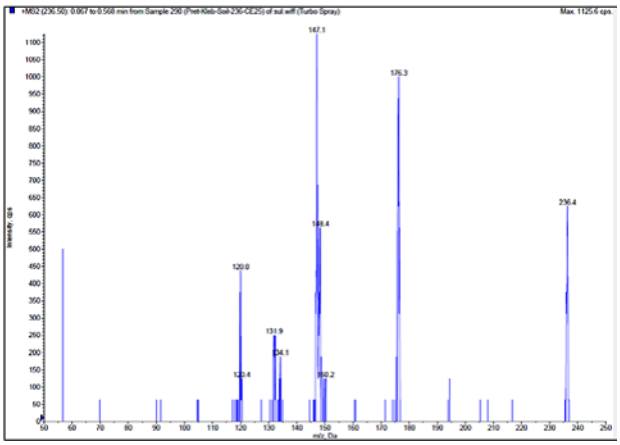

Fig. 7: Mass spectrum of 2', 6'-diethyl-*N*-(propoxyethyl) aniline (V).

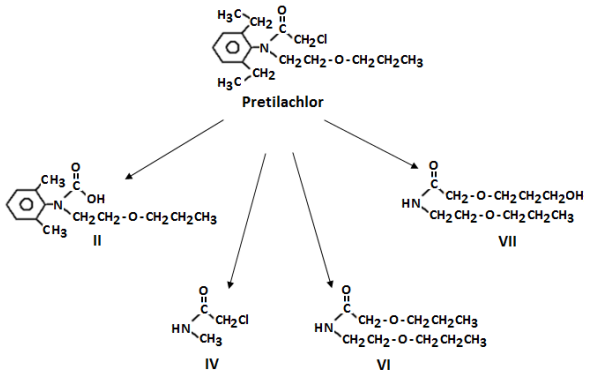

Fig. 8: Degradation of pretilachlor by *Klebsiella species* in water with the formation of four metabolites.

In water: The sterilized water containing pretilachlor was incubated with *Klebsiella* sp. After 20 days of incubation media was extracted with ethyl acetate. The extract was

then cleaned up and analyzed by LC-MS/MS. Four metabolites were isolated and characterized by their respective mass spectra (Fig. 8). Metabolites, viz. 2',6'-dimethyl-*N*-(propoxyethyl) hydroxyl anilide (II) and chloro-*N*-methyl acetamide (IV) were already characterized and described.

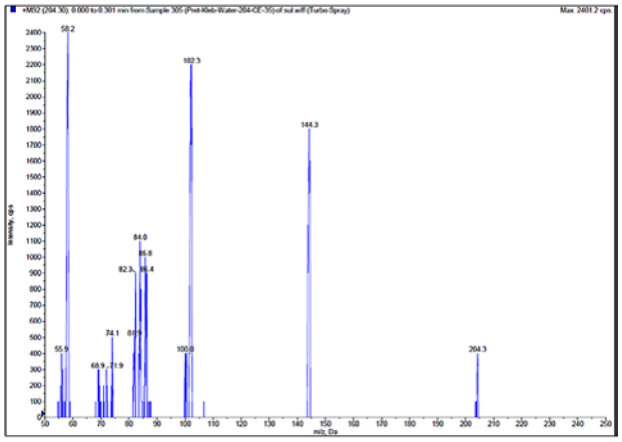

Fig. 9: Mass spectrum of 2-propoxy-*N*-(propoxyethyl) acetamide (VI).

Metabolite VI: In the mass spectrum (Fig. 9) there is no fragment at either 105 or 133 indicating the absence of a substituted benzene ring of pretilachlor. The molecular weight of chlorine less substituted aliphatic amide chain moiety is 144. The substitution of a proton of the acetamide methyl group by a propoxy group gives a compound of molecular weight 203 amu. The peak at 204 is the protonated molecular ion. The peak at M/Z 74 is of methyl propyl ether and that at M/Z 102 is of 2-propoxy acetamide. Combining these fragment ions (Fig. 6) the structure of the molecule is proposed as 2-propoxy-*N*-(propoxyethyl) acetamide.

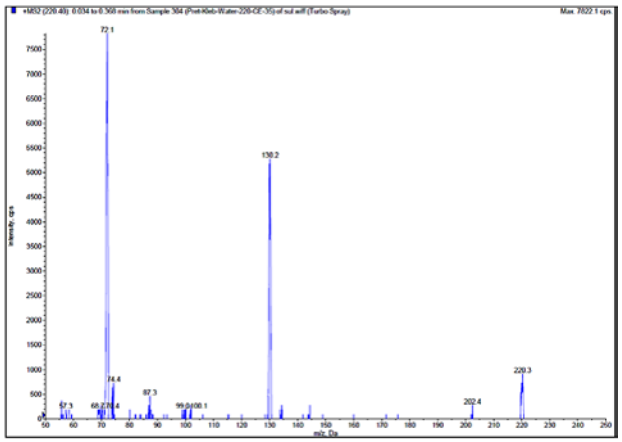

Fig. 10: Mass spectrum of 2-hydroxypropoxy-*N*-(propoxyethyl) acetamide (VII).

Metabolite VII: In this spectrum (Fig. 10) also, there is no fragment at either 105 or 133 indicating the absence of a substituted benzene ring of pretilachlor. The peaks at M/Z 72 and 87 are due to the fragments CH_3-CH_2-NH-CO- and -CH_2-CH_2-O-CH_2-CH_2-CH_3. Combining these two fragments we get another fragment ion of a N-substituted amide at M/Z 130, which on the addition of a propoxymethyl moiety to the carbonyl carbon gives a fragment at M/Z 202. The hydroxylation of the fragment gives a peak at M/Z 220, which is the protonated molecular ion peak. The structure of the molecule is proposed as 2-hydroxypropoxy-*N*-(propoxyethyl) acetamide.

Degradation of pretilachlor by Aspergillus flavus-

The degradation of pretilachlor by the fungus *Aspergillus flavus* in different environments was monitored by TLC and the products were analyzed by LC-MS/MS and characterized by their molecular ions and fragmentation patterns. A number of degradation products of pretilachlor were isolated from different phases, viz. media, soil and water incubated with *Aspergillus flavus*. Pattern of degradation of pretilachlor by the fungus *Aspergillus flavus* can be postulated from the structures of the degradation products.

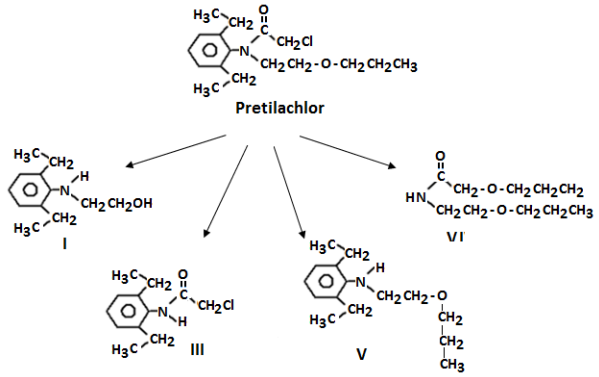

Fig. 11: Degradation of pretilachlor by *Aspergillus flavus* in media with the formation of four metabolites

In the media: Degradation products 2',6'-diethyl-*N*-(2-hydroxyethyl) aniline (I), 2-chloro-2',6'-diethyl acetanilide (III), 2',6'-diethyl-*N*-(propoxyethyl) aniline (V) and 2-propoxy-*N*-(propoxyethyl) acetamide (VI) were isolated from the *Aspergillus flavus*-incubated media and characterized by their respective mass spectra obtained from LC-MS/MS (Fig. 11). Their fragmentation patterns were previously described.

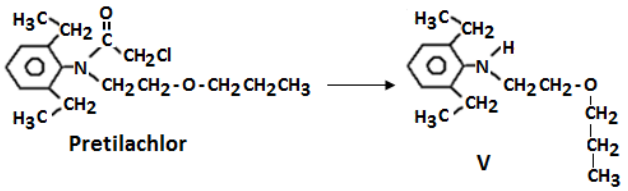

Fig. 12: Degradation of pretilachlor by *Aspergillus flavus* in soil with the formation of a metabolite.

In soil: The only degradation products 2', 6'-diethyl-*N*-(propoxyethyl) aniline (V) was isolated from the *Aspergillus flavus* incubated soil and characterized by their respective mass spectra obtained from LC-MS/MS (Fig. 12). Their fragmentation patterns were described in the previous section.

In water: A couple of degradation products, viz. 2-propoxy-*N*-(propoxyethyl) acetamide (VI) and 2-hydroxypropoxy-*N*-(propoxyethyl) acetamide (VII) were isolated from the *Aspergillus flavus* incubated media and characterized by their respective mass spectra obtained from LC-MS/MS. Their fragmentation patterns were previously.

In the media: Pretilachlor was incubated with the consortium of fungi in media and within 27 days of incubation the entire amount of pretilachlor was degraded. No major metabolites were detected in the Q1 chromatogram obtained from the LC-MS/MS analysis.

IV. DISCUSSION

The proposed study was carried out with an objective to assess the fate of pretilachlor under the influence of microbes isolated from rice field of soil and water. During this study some microbes of beneficial nature which can degrade pretilachlor were isolated and characterized. The outcome of the study indicated that these microbes can even survive in the minimal broth having pretilachlor at the level 200 mg per 200 ml medium. They degraded the herbicide to harvest energy. These findings reveal that the enzymes involved in these transformations can be utilized to detoxicate the soil and water from pretilachlor toxicity. Even, the gene responsible for the production of these useful enzymes can be exploited.

Biodegradation, both in soil and in media by *Klebsiella* sp. and *Aspergillus flavus* indicated that the appropriate consortium of can remove pretilachlor herbicide from soil and water. This study aimed at Optimization of microorganisms and identification of ideal conditions for improved herbicide degradation and bioremediation. Best degradation of pretilachlor was done by *Aspergillus flavus* and *Klebsiella species*. The degradation product of pretilachlor by *Klebsiella* in media metabolite –I, 2',6'-diethyl-*N*-(2-hydroxyethyl) anilinen, metabolite-II 2',6'-dimethyl-*N*(propoxyethyl) hydroxyl anilide, metabolite-III 2-chloro-2',6'-diethyl acetanilide, metabolite-IV chloro-*N*-methyl acetamide, in soil metabolite-V 2',6'-diethyl-*N*-(propoxyethyl) aniline, in water metabolite-VI 2-propoxy-*N*-(propoxyethyl) acetamide. *Klebsiella* is a well known pesticide degrader in the soil. In general, microbial degradation taken place through enzymatic hydrolysis, mostly by co-metabolism process. Due to the uniqueness in its structure, pretilachlor does not allow such easy hydrolysis chemically. But *Klebsiella* did the job of degrading pretilachlor by other different ways.

There are two substitutions in the aniline-N of pretilachlor-one is chloroacetyl group and the other is propoxyethyl group isolated from *Aspergillus* flavus. Pretilachlor suffered an oxidative dechloromethylation followed by two successive demethylation forming the metabolite 2', 6'-dimethyl-*N*-(propoxyethyl) hydroxyl anilide (II). The elimination of chloroacetyl group led to the formation of 2', 6'-diethyl-*N*-(propoxyethyl) aniline (V). This formation of V may be through a step-wise enzymatic reactions via the formation of hydroxyl anilide derivative. Product 2', 6'-diethyl-*N*-(2-hydroxyethyl)aniline (I) was generated from the metabolite V through ether hydrolysis. The cleavage of aliphatic methylene C-N leads to the formation of 2-chloro-2', 6'-diethyl acetanilide (III). The dissociation of substituted N from aromatic moiety generates an intermediate, which is not identified. The rearrangement of this intermediate leads to the formation of 2-propoxy-*N*-(propoxyethyl) acetamide (VI) and 2-hydroxypropoxy-*N*-(propoxyethyl) acetamide (VII) and its further degradation generated chloro-*N*-methyl acetamide (IV). The product found above where nontoxic and thus the toxicity of soil and water was minimized. The degradation of pretilachlor was quite good for 15 days by the organisms. However 45 days of treatment by these microbes was quite effective in the degradation of this herbicide. The residual toxicity of pretilachlor herbicide used for the control of weed can be removed by the fungal and bacterial consortium and can be recommended to the farmers using this herbicide.

V. CONCLUSION

The study concluded the degradation of pretilachlor under the influence of microbes isolated from rice soil and water. During this study some microbes of beneficial nature which can degrade pretilachlor were isolated and characterized. The outcome of the study indicated that these microbes can even survive in the minimal broth having pretilachlor at the level 200 mg per 200 ml media. They degraded the herbicide to harvest energy. These findings reveal that the enzymes involved in these transformations can be utilized to decontaminate soil and water from pretilachlor contamination. Even, the gene responsible for the production of these useful enzymes can be exploited. Biodegradation, both in soil and in the media by microbes indicated that the appropriate consortium of microbes can remove pretilachlor herbicide from soil and water. These studies conclude ideal conditions for improved herbicide degradation by microbes. It will also generate important information on the development of bioremediation technology using the selected microbes, besides screening and characterization of pretilachlor degrading microbes.

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ACKNOWLDGEMENT

The authors would like to thank Supervisor, Dept. of Botany & Microbiology, Govt. Home Science College, Jabalpur for special and experienced guidance and motivation. Her ever-helping attitude, deep knowledge and readiness to provide proper facility and quality supervision have boosted me to complete the present work. I would like to thank all professors of the M.H. College of Home Science and Science for Women, Jabalpur for providing research facilities. I express my sincere thanks to scientist of D.W.S.R. Jabalpur (M.P.) and all the Professors of Department of Biological Science for their constant help and worth suggestions.