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Practical Case Study

BIODEGRADATION OF DIAZINON AND METHOMYL PESTICIDES BY WHITE ROT FUNGI FROM SELECTED HORTICULTURAL FARMS IN RIFT VALLEY AND CENTRAL KENYA

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Abstract: The aim was to investigate the potential for biodegradation of methomyl and diazinon (pesticides) by white rot fungi through enrichment and isolation of methomyl and diazinon biodegraders from horticultural soils. Five white rot fungal isolates labeled WR1, WR2, WR4, WR9 and WR15 were cultured in a medium containing methomyl and diazinon pesticides as the only carbon source and incubated at 28^0C and monitored for biodegradation at intervals of 10 days for a period of 100 days. Gilson HPLC system was used for the separation and quantification of the pesticides. The pesticide methomyl was detected at 4.9 minutes while the methomyl metabolite was detected at 4.1 minutes. Diazinon was detected at 11 minutes while the diazinon metabolites; diazoxon and oxypyrimidine were detected at 2.3 and 2.6 minutes. The rate of biodegradation of pesticides was much higher for mixtures of fungi (approximately 50 days) compared to individual isolates (60- 100 days) for diazinon while methomyl was 22-25 days. This shows that fungal mixtures in soil fasten the rate of biodegradation of pollutants compared to individual isolates. The isolates are good biodegraders due to their ability to degrade methomyl or diazinon hence making the isolates good agents for bioremediation. The authors recommend more studies to be done in the fields before their potential application by farmers.

Keywords: Biodegradation, HPLC, Metabolite and White rot fungi isolates

INTRODUCTION

One of the major environmental problems facing the world today is the contamination of soil, water, and air by toxic chemicals. Eighty billion pounds of hazardous organopollutants are

produced annually in agricultural farms and only 10% of these are disposed of safely [23]. Certain hazardous compounds, such as methomyl and diazinon, are persistent in the environment and are known to have carcinogenic and/or mutanogenic effects. It can cost up to approximately $1 trillion to decontaminate toxic waste sites in the agricultural farms using traditional waste disposal methods such as incineration and land filling [23]. Due to the magnitude of this problem and the lack of a reasonable solution, a rapid, cost-effective, ecologically responsible method of cleanup is greatly needed. Bioremediation or utilizing microorganisms to degrade toxic organopollutants is an efficient, economical approach that has been successful in laboratory studies [7, 19].

The mechanisms for the clean up of pesticides in soil such as chemical treatment, volatilization and incineration have met public opposition, because of problems such as large volumes of acids and alkalis which are produced and subsequently must be disposed off, also the potentially toxic emissions and the elevated economic costs [15]. Overall, most of these physical-chemical cleaning technologies are expensive and rather inefficient. These clean-up methods do not suit large farms since only small soil samples are required and they are done in the laboratories and hence require a lot of resources because the contaminated soil has to be excavated at a site and moved to a storage area where it can be processed [14, 21]. Due to environmental concerns associated with the accumulation of pesticides in food products and water supplies there is a great need to develop safe, convenient and economically feasible methods for pesticide remediation [34, 16]. For this reason several biological techniques involving biodegradation of organic compounds by microorganisms like bacteria and fungi (white rot fungi) have been developed [25, 17].

Expansion and intensification of agricultural and industrial activities in recent decades has led to pollution of soil and groundwater with pesticides and many treatment processes have been developed to reduce the environmental impacts of this contamination. In contaminated soils, microorganisms are more commonly found in mixtures. Very few studies have examined the degradation of pesticides using mixtures of microorganisms in soils. Moreover, there are hardly any studies on the use of mixtures of white rot fungi to clean-up pesticides.

Diazinon is found in all environmental compartments and given adequate time, it will be degraded by abiotic and biotic processes so that the parent compound is not persistent [12]. Degradation products of diazinon include diazoxon and oxypyrimidine [9]. Oxypyrimidine is the main soil and water degradate of diazinon. Diazinon can be converted to diazoxon in the atmosphere via ultraviolet [26, 27]. Diazinon released to surface waters or soil is subject to volatilization, photolysis, hydrolysis, and biodegradation. Diazinon has a relatively short half-life in water, ranging from 70 hours to 12 weeks depending on pH, temperature, and sunlight as well as the presence of microorganisms while in soil it is influenced by the pH conditions in the soil and the soil type [27]. In agricultural soils, methomyl is rapidly mineralized to carbon dioxide. No other degradation compound is observed in soil in significant amounts [28, 31]. Methomyl oxime is a minor transient degradation product observed at a maximum of 2.9% of applied active ingredient.

White rot fungi is a physiological grouping of fungi that can degrade lignin. Four main genera of white rot fungi have shown potential for bioremediation: *Phanerochaete, Trametes, Bjerkandera*, and *Pleurotus* [13]. These fungi cannot use lignin as a source of energy, however, and instead require substrates such as cellulose or other carbon sources. Also, the branching, filamentous mode of fungal growth allows for more efficient colonization and exploration of contaminated soil [20]. The main mechanism of biodegradation employed by this group of fungi, however, is the lignin degradation system of enzymes. These extracellular lignin modifying enzymes (LMEs) have very low substrate specificity so they are able to mineralize a wide range of highly recalcitrant organopollutants that are structurally similar to lignin [8, 18, 22, 32]. The

three main LMEs are lignin peroxidase, Mn-dependent peroxidase, and laccase. All three of these enzyme groups are stimulated by nutrient limitation [18, 2]. The objectives of this work were to evaluate the ability of five white-rot fungi isolates (WR1, WR2, WR4, WR9 and WR15) to degrade methomyl and diazinon pesticides.

MATERIALS AND METHODS

Chemicals

Diazinon and methomyl were purchased from Sigma-Aldrich Chemical Company. All other chemicals, bacterial media and reagents were purchased from Oxoid limited- England, Scharlau Chemie- South Africa, Himedia laboratories and PVT limited- India. All the solvents and chemicals were high purity grade reagents.

Soil particle washing and plating

Sampling was done using stratified random sampling method from two regions in Kenya; Rift-valley region and Mt. Kenya region. Soil cores of 2.5 cm diameter were taken to 5 cm depth after the litter layer was removed. The geographical regions formed four strata and from each region, two plots were identified by simple randomization. Similar sampling was done 100 m away from the farms to act as controls [1,3]. Samples were stored at 4^0C until processing, in most cases within 2 days. Approximately 5 g fresh weight (2.5 to 4.5 g dry weight) of each soil sample was added to 500 ml of sterile 0.1% (wt/vol) sodium pyrophosphate in 1-liter mason jars. These were gently shaken end-to-end on a platform shaker for 1 h at 48^oC to disperse soil clumps and colloids [4. 5, 6]. The entire suspension was poured through stacked 20 cm diameter soil sieves (Newark wire cloth) of 250 mm (no. 60) and 53 mm (no. 270) mesh and rinsed through with a brief shower of cold tap water. Particles remaining on the 53mm mesh sieve were then washed for 5 min under this shower at a flow rate of approximately 20 liters/min. Remaining solids were collected at one edge of the sieve, and the sieve was tilted to separate suspended organic particles from settled mineral particles. One milliliter of a dense suspension of the organic particles was picked up in a sterile broad-bore pipette tip (Gilson P-1000). This suspension was diluted in sterile distilled water to 10^2, and 0.4 ml of this dilution was spread onto each of 20 petri dishes of lignin-guaiacol-benomyl agar. Sieves were rinsed with water and sterilized in 70% ethanol between samples [5, 10].

Isolation and identification of white rot fungi

After one day of inoculation, the Petri dishes were packed in their plastic sleeves and incubated at 28^0C for 2 weeks before making isolations. At this time, plates were scanned for colonies that caused reddening of the guaiacol by the action of laccase or peroxidase. These colonies were examined microscopically (at x40 and x100) for the presence of conidia or clamp connections. Plates were screened again after 4 and 6 weeks. At each screening, colonies of putative basidiomycetes were isolated onto malt-yeast agar containing chloramphenicol and tetracycline. In order to identify putative white rot fungi isolates, cultures were considered to be basidiomycetes if they showed clamp connections at septa or positive staining with diazonium blue B ($ZnCl_2$ complex of tetrazotized *o*-dianisidine; Sigma) [30].

Minimal mineral media for methomyl and diazinon liquid cultures

Mineral medium MMN (mineral medium without nitrogen and carbon) was derived from mineral medium MMO by elimination of all nitrogen and carbon. MMN medium contained 1,40 mg

of Na_2HPO_4, 1,36 mg of KH_2PO_4, 98.5 mg of $MgSO_4$, 5.88 mg of $CaCl_2 \cdot 2H_2O$, 1.16 mg of H_3BO_4, 2.78 mg of $FeSO_4 \cdot 7H_2O$, 1.15 mg of $ZnSO_4 \cdot 7H_2O$, 1.69 mg of $MnSO_4 \cdot H_2O$, 0.38 mg of $CuSO_4 \cdot 5H_2O$, 0.24 mg of $CoCl_2 \cdot 6H_2O$, 0.10 mg of MoO_3, and 3.2 mg of EDTA in 1 liter of distilled water. The liquid mineral medium was supplemented with 50 ppm and 12.5 ppm of methomyl and diazinon respectively.

Isolation of white rot fungi using methomyl and diazinon as the only sole carbon source

Soil samples (5 g) contaminated with diazinon and methomyl were used to inoculate baffled Erlen–meyer flasks containing 50 ml mineral medium supplemented with diazinon and methomyl as sole carbon source. Flasks were incubated at 28^{o}C with shaking (1200 rpm) in the dark. After approximately 7 days, samples from these cultures were spread-plated on mineral salts agar containing 12 ppm diazinon and 2M methomyl. Isolates that showed fast growth on plates were selected for further analysis.

White rot fungi isolates growth in liquid culture

Isolates were precultured in baffled erlenmeyer flasks containing mineral salts medium with the above mentioned concentrations of pesticides. Flasks were incubated at 30^{0}Cwith shaking (1200 rpm) in the dark. Growth was monitored as changes in OD600 (Shimadzu, Japan). When growth had occurred, the flasks contents were centrifuged the cell pellets were then washed in fresh sterile medium 4 times before addition to mineral medium and the pesticides. After 10 days interval 2.5 ml samples were removed and cell growth monitored. Two controls were performed: uninoculated medium with the pesticides and medium without the pesticides inoculated with the isolates [11].

HPLC analysis of pesticide degradation

The degradation of the pesticides was monitored by high performance liquid chromatography. (Shimadzu HPLC class *VP* series) with two LC – 10 AT *VP* pumps (Shimadzu), variable wavelength UV detector SPD10*VP* (Shimadzu), CTO-IOAS *VP* column oven (Shimadzu), (Shimadzu) and a reverse phase C-18 column,250 x 4.6mm,fitted with a C-18 silica reverse phase guard column was used. (Fisher Scientific, Fairlawn, N.J) The HPLC system was equipped with software class *VP* series ss420x (Shimadzu). The mobile phase components acetonitrile and degassed water were pumped from the solvent reservoir to the column at a flow rate 1 mL/min. The column temperature was maintained at 27^{0} C. 20 µL of sample was injected using Rheodyne syringe (Model 7202, Hamilton). Diazinon and methomyl were identified by their retention times and peaks corresponding to reference standards.

DNA extraction

This was done to identify the fungal isolates. Each of the 16 isolates was grown on malt extract agar for 7 days. Total genomic DNA of the isolates was extracted from these cells in duplicate using two lysis buffers as solution A (50mM Tris pH 8.5, 50mM EDTA pH 8.0 and 25% sucrose solution) and solution B (10mM Tris pH 8.5, 5mM EDTA pH 8.0 and 1% SDS). The cells were scrapped asceptically using a sterile surgical blade taking care not to pick the media. These were crushed separately in 200µl solution A using sterile mortar and pestle, and resuspended in 100µl of solution A. This was followed by addition of 30µl of 20mg/l Lysozyme and 15µl of RNase, gently mixed and incubated at 37^{o}C for two hours to lyse the cell wall.600µl of Solution B was then added and gently mixed by inverting the tubes severally, followed by the addition of 10µl of Proteinase K (20mg/l) and the mixture incubated at 60^{o}C for 1hour. Extraction followed

the phenol/chloroform method.The presence of DNA was checked on 1% agarose and visualized under ultraviolet by staining with ethidium bromide. The remaining volume was stored at -20^{o} C. The genomic DNA was used as templates for subsequent PCR amplification

PCR amplification of 18srDNA

Total DNA from each isolate was used as a template for amplification of the 18S rRNA genes. Nearly full-length 18S rRNA gene sequences were PCR-amplified using fungal primer pair Fung5f forward 5'-GTAAAAGTCCTGGTTCCCC-3' and FF390r reverse, 5'-CGATAACGA ACGAGA CCT-3'. Amplification was performed using Peqlab primus 96 PCR machine. Amplification was carried out in a 40µl mixture containing 5µl of PCR buffer (×10), 3µl dNTP's (2.5mM), 1µl (5 pmol) of Fung5f forward primer, 1µl (5pmol) of FF390r reverse primer, 0.3µl taq polymerase, 1.5µl of template DNA and 26.2µl of water. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles repeated for 36 cycles: Initial activation of the enzyme at 96^{o}C for five minutes, denaturation at 95^{o}C for 45 seconds, primer annealing at 48^{o}C for 45 seconds, chain extension at 72^{o}C for 1.30 minutes and a final extension at 72^{o}C for 5 minutes. Amplification products (5µl) were separated on a 1% agarose gel in 1× TBE buffer and visualized under ultraviolet by staining with ethidium bromide.

Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's s instructions. Five volumes of buffer PB (Qiagen, Germany) was added to 1 volume of the PCR sample and thoroughly mixed. The QIAquick spin column was placed in a 2ml collection tube; the sample was applied to the QIAquick column to bind the DNA, and then centrifuged for 60 seconds at 13000 rpm. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 740 µl buffer PE was added to the QIAquick column and centrifuged for one minute. The flow-through was discarded and the column centrifuged again for an additional one minute at 13000rpm to remove residual ethanol from buffer PE. The Qiaquick column was placed in a 1.5ml micro centrifuge tube and 30µl of buffer EB (10mM Tris-Cl, pH 8.5) added to elute DNA. The tubes were then centrifuged for one minute, the spin column removed and DNA stored at –20^{o}C for application.

Restriction digestion of PCR products

PCR reaction products were digested directly without further purification with restriction endonucleases to obtain RFLPs; each sample was digested with *Taq*I. Per each 20-µl restriction digest, 10 µl of unpurified, amplified PCR reaction was mixed with the appropriate restriction reaction buffer and 10 µl of the appropriate enzyme and then incubated for 6 h at 65°C for the *Taq*I digests. Restriction fragments were separated by electrophoresis in 2% (wt/vol) and 2.5% (wt/vol) Sepharide Gel Matrix (Gibco-BRL) in 1× TAE (40 mM Tris acetate, 1 mM sodium EDTA) with EtBr at 100 ng/ml in the gel and running buffer. DNA bands were visualized by fluorescence under UV light and photographed.

Phylogenetic analysis

The forward 18S rRNA gene sequences of the 16 white rot fungi isolates were viewed and edited using Chromas software package (www.technelysium.com.au). They were then aligned using Bioedit sequence alignment editor software package to provide full sequences of about

1500 nucleotide bases. The sequences were compared to sequences in the public databases with the BLAST search program on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 18S rRNA gene sequences. The ARB database software package was also used to align and identify the closely related fungal 18S rRNA gene sequences. The 18S rRNA gene sequences of the isolates and those of the closely related fungi were then aligned and processed to produce Phylogenetic trees using MEGA software package (www.megasoftware.net). The evolutionary history was inferred using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 1000 replicates was used. The trees were drawn to scale, with branch lengths being in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset.

RESULTS AND DISCUSSION

In these study, two major findings in biodegradation of contaminated soils namely; the ability of white-rot fungi in Methomyl or Diazinon degradation and the use fungal consortia in promotion of enhanced degradation were reported. A total of sixteen white rot fungi were isolated with ease from soils with history of diazinon and methomyl pesticide contamination. This finding is important as it suggests a possible use of the white rot fungi in metabolizing of methomyl and diazinon. This finding agrees with the findings of Sasek (24) who reported the ability of a white rot fungus *Phanerochaete chrysosporium* to metabolize a number of various important environmental pollutants. The need to remediate contaminated sites has led to the development of new technologies that emphasize the destruction of the contaminants rather than the conventional approach of disposal [7] hence possible use of white rot fungi may be an important factor in this solution.

Morphological characterization of white rot fungal isolates

Plate 1 shows a red color at the back side of a plate containing the medium lignin guaiacol benomyl agar with low concentration of lignin around the point of growth, while Plate 2 shows the front side of the same plate which is characterized by the growth of mycelia. Plate 3 shows a plate with high concentration of lignin.

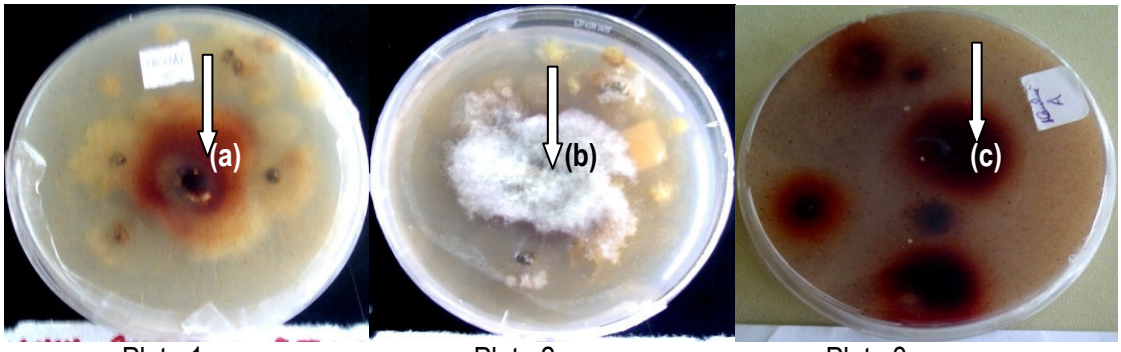

Plate 1 Plate 2 Plate 3

Plate 1,2,3: Isolates producing laccase or peroxidase on lignin-guaiacol-benomyl agar are readily located by the dark and bright red zone beneath their colonies as indicated by the arrows; (a)Low concentration of lignin (1g) bright Reddening of the media; (b)Fungal growth; (c)Increased lignin concentration (2g) thus dark red colour.

Microscopic characterization of white rot fungal isolates

Plate 4 shows clamp connections (arrows) as seen under the microscope at x100 objective lens.

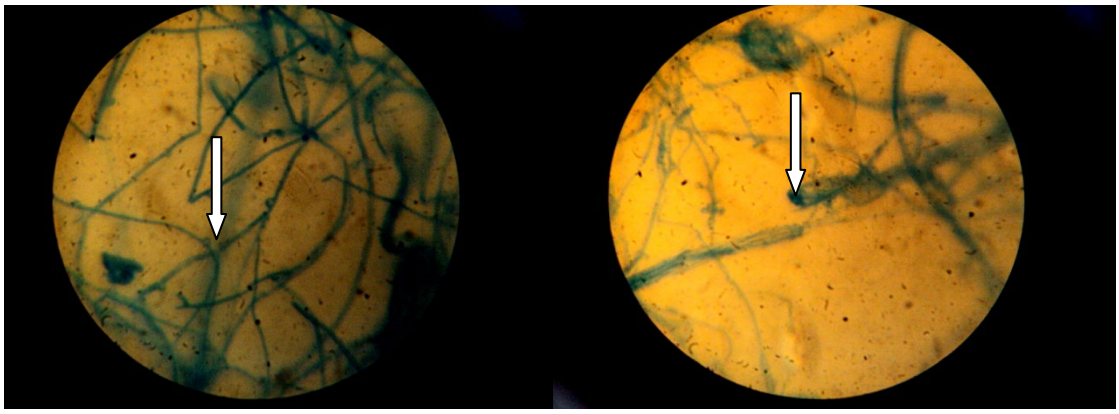

Plate 4: Isolate showing clamp connections (arrow) as seen under the microscope at x100 objective lense.

Plate 5: White rot fungi isolate showing fruiting body (arrow) as seen under x100 objective lense.

Biodegradation profiles of Methomyl by white rot fungi

The results in figure 2 and 3 show that methomyl pesticide at a wavelength of 235nm, it is detected at an hplc runtime of 4.9 minutes while its metabolite at 4.1 minutes. According to Strathmann [28], soil studies demonstrate that methomyl degrades rapidly in aerobic soil to yield carbon dioxide and biologically unavailable and unextractable residue. The minor transient degradation product of methomyl, methomyl oxime, degrades even more rapidly. Strathmann (28) agrees with our results which show that the metabolite peak at 4.1 minutes disappeared over time before degradation of methomyl was achieved. Veignie [32] stated that microorganisms in soil utilize metabolites produced during degradation by other organisms. This was evident when two fungal isolates were mixed, hence accelerating the rate of disappearance of the pesticides as shown in figure 4 where degradation took less than 42 days to be complete for a single isolate. However, when the isolates were mixed (figure 5), degradation took half (22-25 days) the time taken by a single isolate. This finding shows that degradation is much quicker and faster when microbes are used in mixtures compared to when they are used individually.

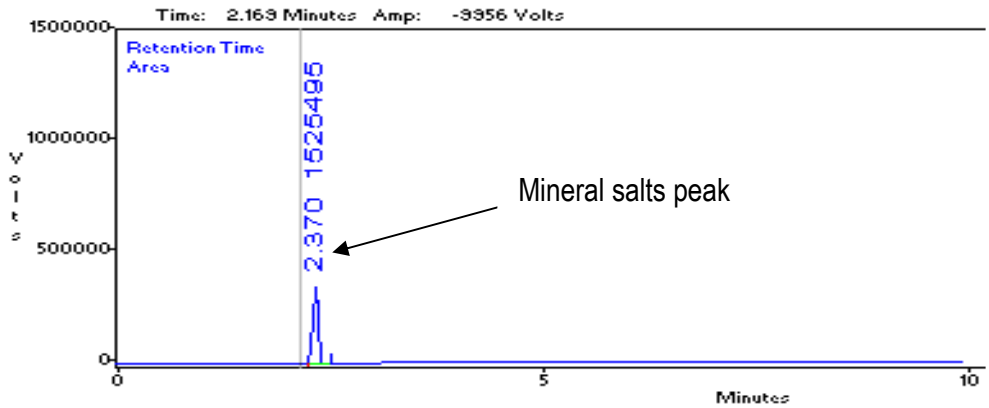

Fig. 1: Profile of Minimal mineral salt medium run in the Hplc at a retention time of 2.3 minutes (1525495) at a flow rate of 1ml/min for a run time of 10 minutes after 0 days

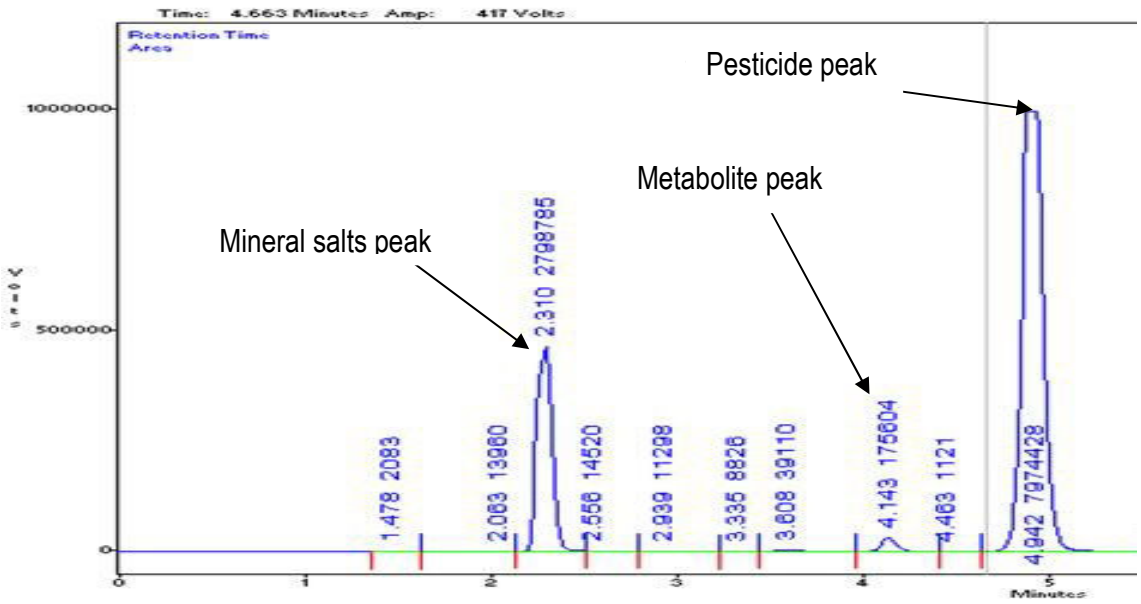

Fig. 2: Methomyl profile showing mineral salt peak at retention time of 2.3 minutes (2798785), metabolite peak at retention time 4.1 minutes (175604) and pesticide peak with a retention time of 4.9 minutes (7974428) after a hplc runtime of 10 minutes for 10 day old WR2 culture of white rot fungi.

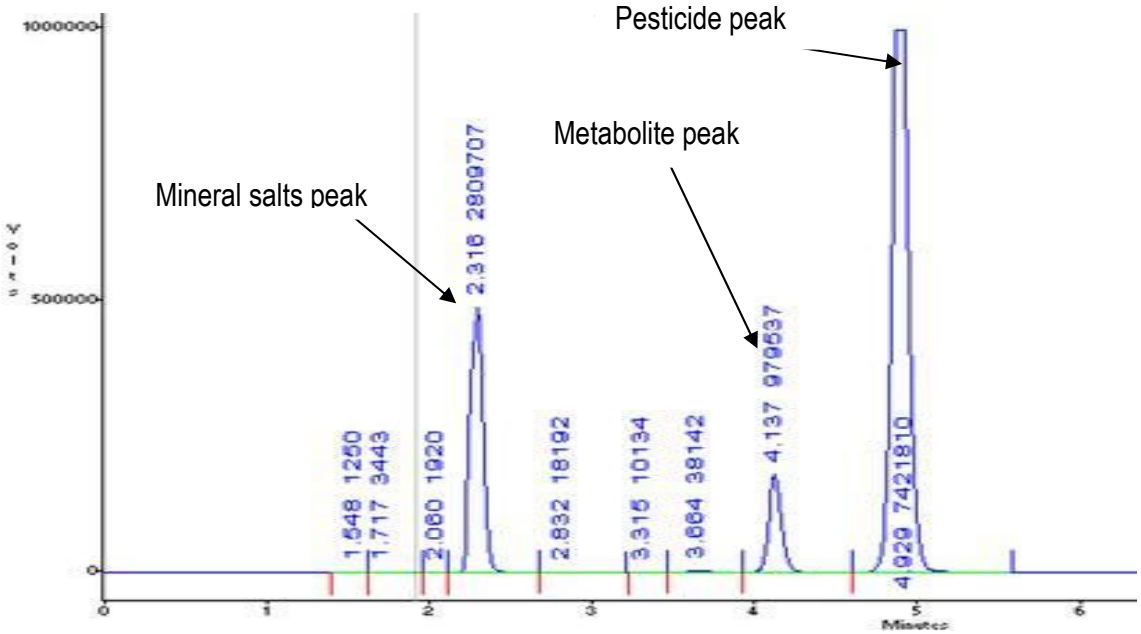

Fig. 3: Methomyl profile showing mineral salt peak at retention time of 2.3 minutes (2809707), metabolite peak at retention time 4.1 minutes (979537) and pesticide peak with a retention time of 4.9 minutes (7421810) after a hplc runtime of 10 minutes for 10 day old WR1 & WR9 cultures of white rot fungi.

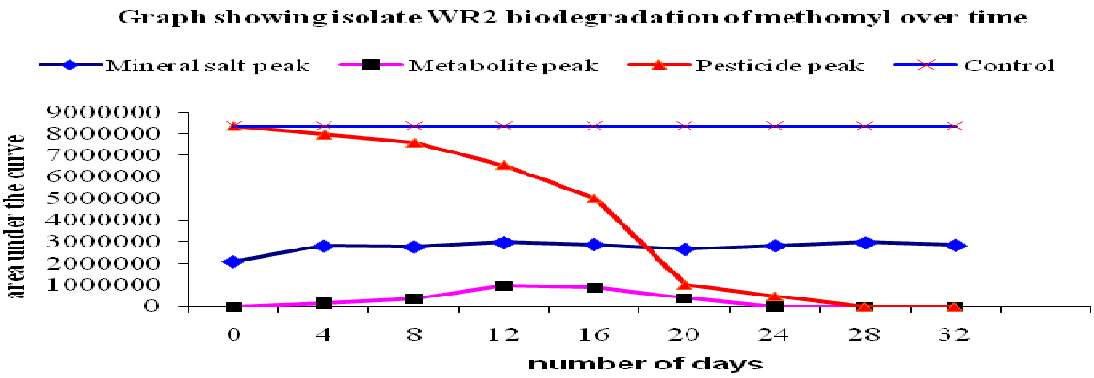

Fig. 4: Methomyl biodegradation profile of isolate WR2 over a period of 40 days

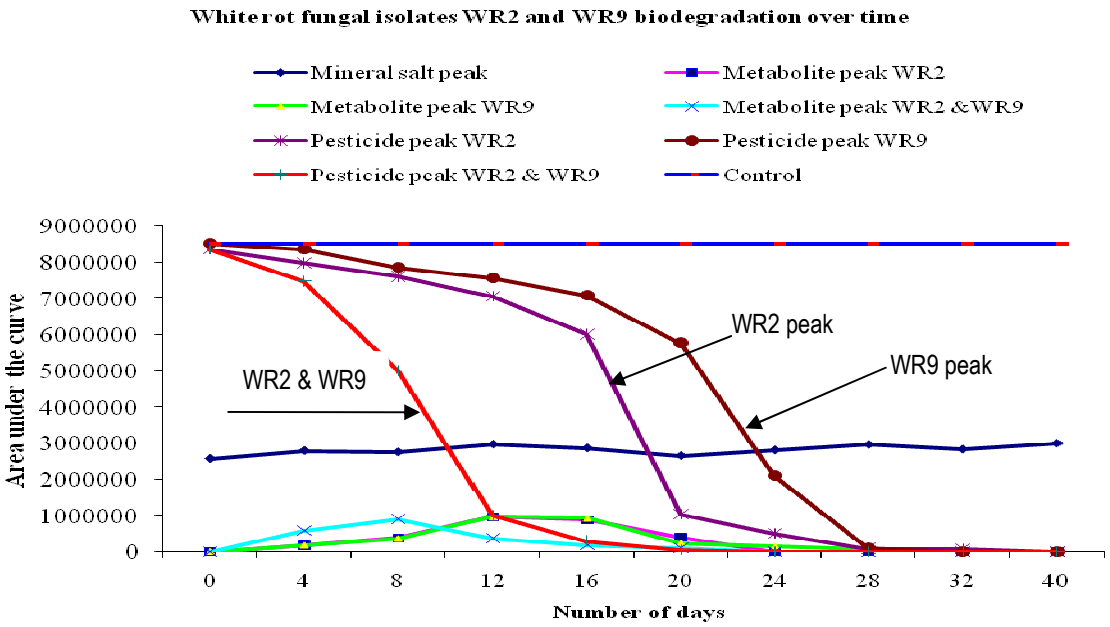

Fig. 5: Methomyl biodegradation profile of a mixture of isolate WR2 & WR9 over a period of time

Biodegradation profiles of Diazinon by white rot fungi

In the results diazinon at a wavelength of 254nm, was detected at a retention time of 11 minutes and metabolites at 2.6 and 2.8 minutes as shown in figure 6 and 7. This agrees with the work of Sethunathan [27] who stated that "Degradation products of diazinon include diazoxon and oxypyrimidine". From the results, the two metabolite peaks represent both diazoxon and oxypyrimidine. According to Suet [29], diazinon released to surface waters or soil is subject to volatilization, photolysis, hydrolysis, and biodegradation. In the study, the area under the curve of the pesticide peak for the control reduced slightly, an observation that would easily be attributed to photolysis. It is important to note that biodegradation, primarily under aerobic conditions, is a major fate process for diazinon associated with water and soil, whereas hydrolysis is an important mechanism for degradation, particularly at low pH in water and soil [29].

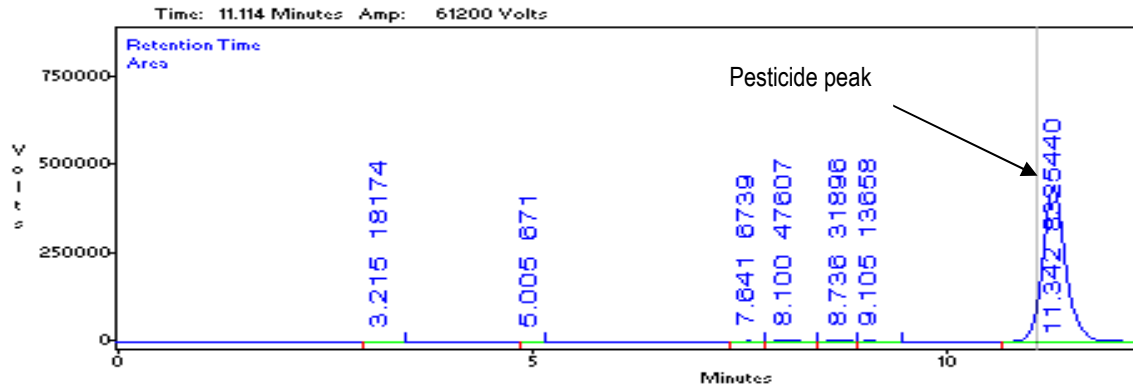

Fig. 6: Profile showing peak of diazinon pesticide with time retention of 11 minutes (8325440).

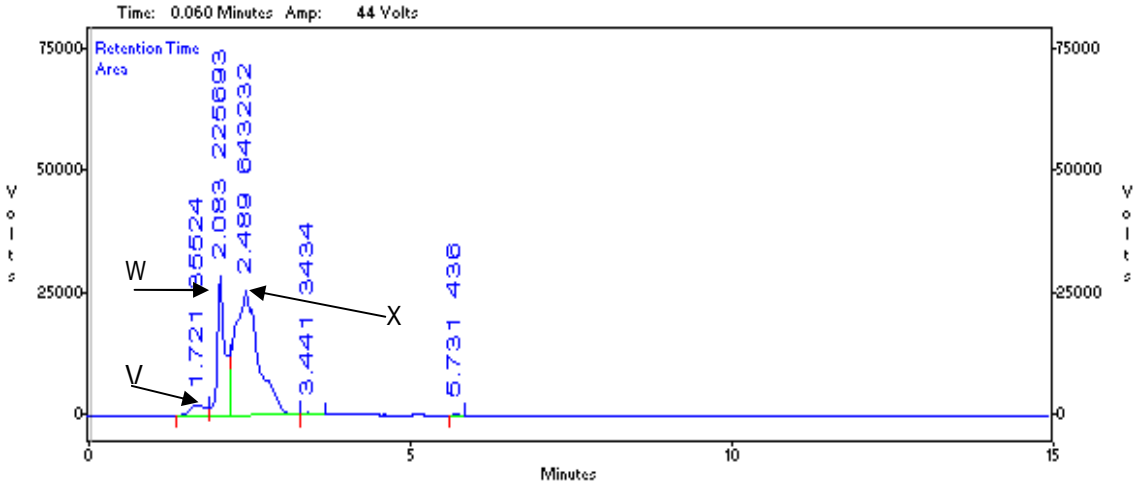

Fig. 7: Diazinon mineral salt peak V, W & X at retention time 1.7 minutes (35524), 2.0 minutes (225693) and 2.4 minutes (643232) respectively.

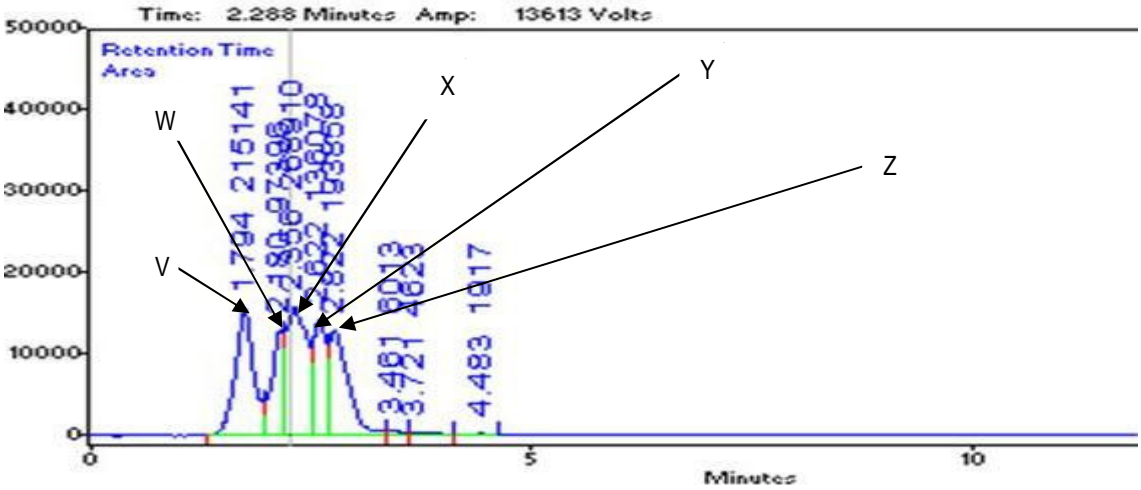

Fig. 8: Profile showing complete biodegradation of the pesticide diazinon by white rot fungi isolate WR15 after 80 days. The mineral salt peaks are V at retention time 1.7 minutes (215141), W retention time 2.1minutes (97396), X retention time 2.3minutes (268910) while the metabolite peaks are; Y retention time 2.6minutes (136078) and Z retention time 2.8 minutes (193858).

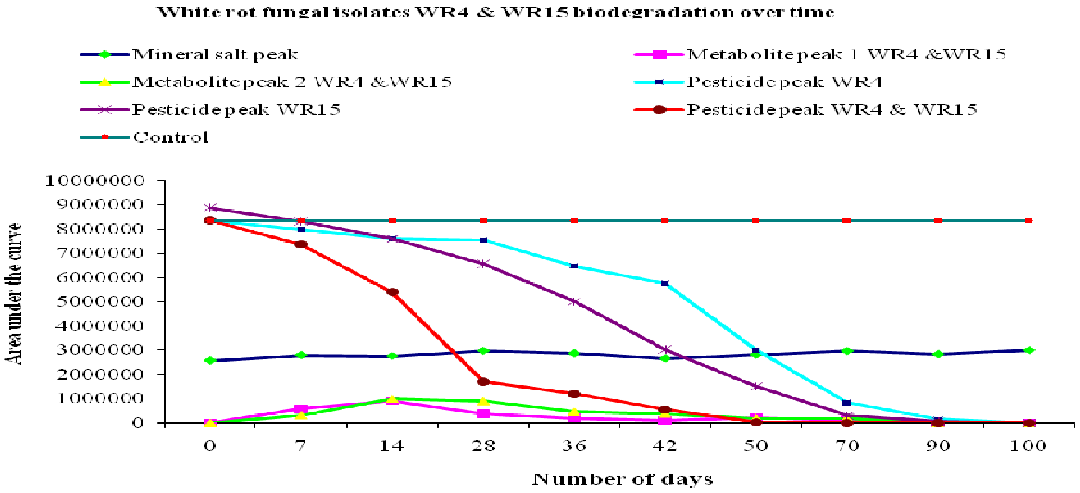

Fig. 9: Diazinon biodegradation profile of white rot fungi isolates WR4 &WR15 over a period of 57 days

The significant fungal growth rates observed with five isolates (WR1, WR2, WR4, WR9 and WR15), after a series of screening in biodegradation studies, is another important finding in this study as it shows an essential component of these fungi in bioremediation. The study of fungal growth rates is very important for extrapolation of the potential colonization capacity in the field as it provides a good indication of the speed at which a fungus is able to colonize and transverse a substrate. Growth rates may also indicate which species may be dominant over a particular substrate; fast growing species have an advantage over slower species as they can reach and utilize resources before their competitors [24]. Therefore, better growth could help the introduced fungi to overcome competition from indigenous soil microorganisms [32]. Since mixtures of microorganisms exist in soil [2], they compete for sources of carbon for their survival by utilizing the available substrates ending up in toxic or less toxic metabolites as by products [23, 33].

Molecular characterization of isolates

DNA extraction and PCR amplification. Extractions of DNA followed by the phenol chloroform method yielded DNA clean enough to amplify by PCR regardless of the starting material being fungal mycelia. All of the phenol chloroform extracted DNA preparations were amplified by PCR (figure 10). PCR amplifications were performed using total DNA isolated from pure cultures of the fungi as the DNA template. Primers Fung 5f and FF390r, Basid001 and Basid-2R+ were used for amplification (figure 11).

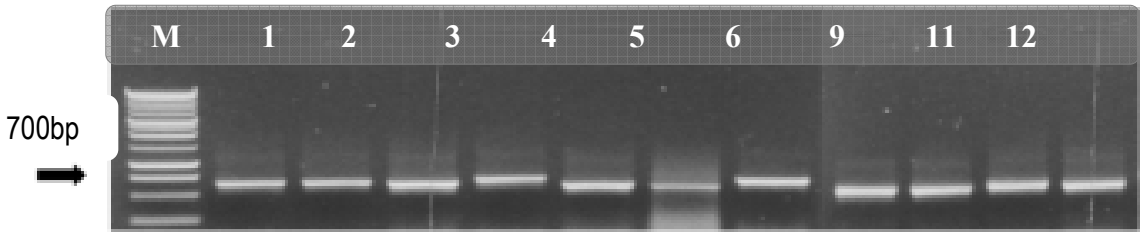

Fig. 10: Amplification of white rot fungal isolates by using the Fung5f forward 5'-GTAAAAGTCCTGGTTCCCC-3' and FF390r reverse, 5'-CGATAACGA ACGAGA CCT-3' primer pair. Fungi genomic DNA was used as a template and the PCR products were separated with 1% EtBr-agarose gel. M; Marker.

Fig. 11: PCR amplification of white rot fungal isolates using two different sets of primers (Fung 5f and FF390r, (Basid-2R+ [5'-TACCGTTGTAGTCTTAACAG-3'] and Basid001 [5'-GCTTTACCACATAAATCTGA-3']).

PCR amplification of nuclear rDNA from total DNA isolated from white rot fungal isolates. Electrophoresis in 1% (wt/vol) agarose in 1× TBE. The two outer lanes contain molecular weight markers. Inner even-numbered lanes contain samples amplified by the primer pair Fung 5f and FF390r, and the odd-numbered lanes contain samples amplified by primers Basid- 2R+ and Basid 001. Lanes 1 and 20, PCR markers (Promega); lanes 2 and 3, WR1; lanes 4 and 5, WR2; lanes 6 and 7, WR4; lanes 8 and 9, WR9; lanes 10 and 11, WR12; lanes 12 and 13, WR15; lanes 14 and 15, WR8; lanes 16 and 17, WR11; lanes 18 and 19, no template DNA (i.e., negative controls).

Restriction digests

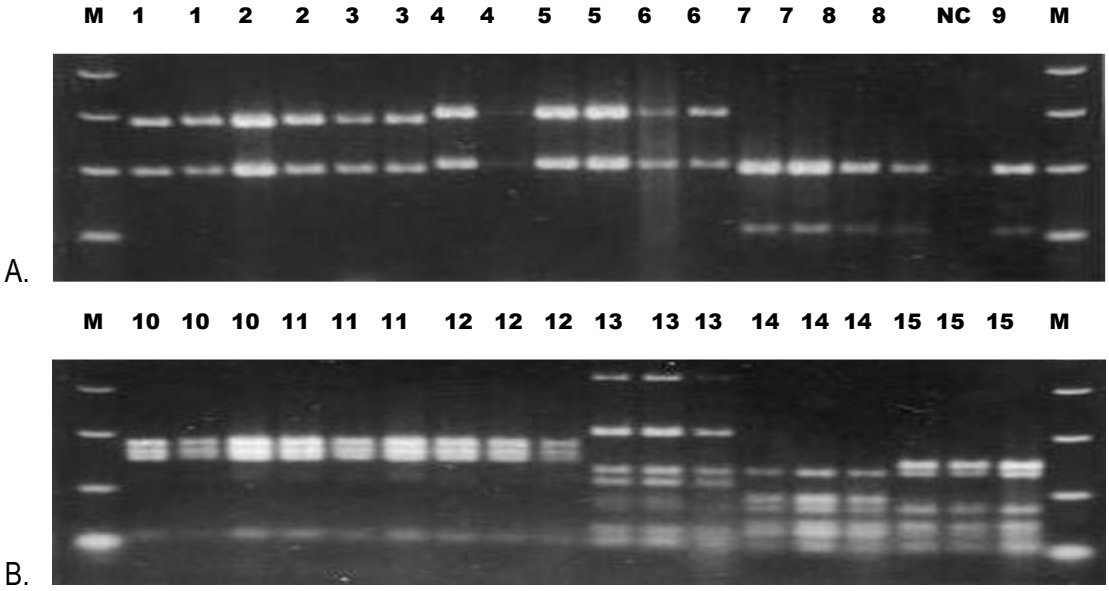

Fig. 12: *Taq*I restriction digests of the PCR product amplified by the primer pair Fung 5f and FF390r from DNA isolated from white rot fungi isolates.

Electrophoresis was done in 1% (wt/vol) Sepharide Gel Matrix (Gibco-BRL) in 1× TAE. The two outer lanes contain molecular weight markers. Each group of two inner lanes represents the *Taq*I digests for one fungal isolate amplified from DNA respectively for A while each group of three inner lanes represents *Taq 1* digests for one fungal isolate amplified from it's DNA for B. (A) Lanes 1 and 20, PCR markers (Promega); lanes 2 to 3, WR2; lanes 4 to 5, WR3; lanes 6 to 7,

WR4; lanes 8 to 9, WR5; lanes 10 to 11, WR6; lanes 12 to 13, WR7; lanes 14 to 15, WR10; lanes 16 to 17, WR11; lane 18, Negative control; lane 19, WR8. (B) Lanes 1 and 20, PCR markers (Promega); lanes 2 to 4, WR13; lanes 5 to 7, WR14; lanes 8 to 10, WR9; lanes 11 to 13, WR15; lanes 14 to 16, WR1; lanes 17 to 19, WR12.

Phylogenetic analysis. DNA was extracted from the isolates, amplified using primers Fung5f forward 5'-GTAAAAGTCCTGGTTCCCC-3' and FF390r reverse, 5'-CGATAACGA ACGAGA CCT-3' primer pair. PCR products were first verified using gel electrophoresis in 1.0 % agarose gel (Figure 10). The PCR products were then purified and the purified product was verified by electrophoresis in 1.0 % agarose gel. The purified PCR products were then sequenced using the above primers. The sequenced products were then blasted against samples at NCBI database at http://www.ncbi.nlm.nih.gov/.

Phylogenetic analysis of 18S rRNA gene sequences of isolate WR2, WR9 and WR4 showed that the three isolates clusters with the genus *Pleurotus* (Appendix 1). This clustering pattern was supported by high bootstrap values of between 96 to 99 % (Figure 13). The isolates had sequence similarity of 99 % to *Pleurotus cornucopiae, Pleurotus eryngii, Pleurotus nebrodensis and Pleurotus ostreatus* among others (Appendix 1)

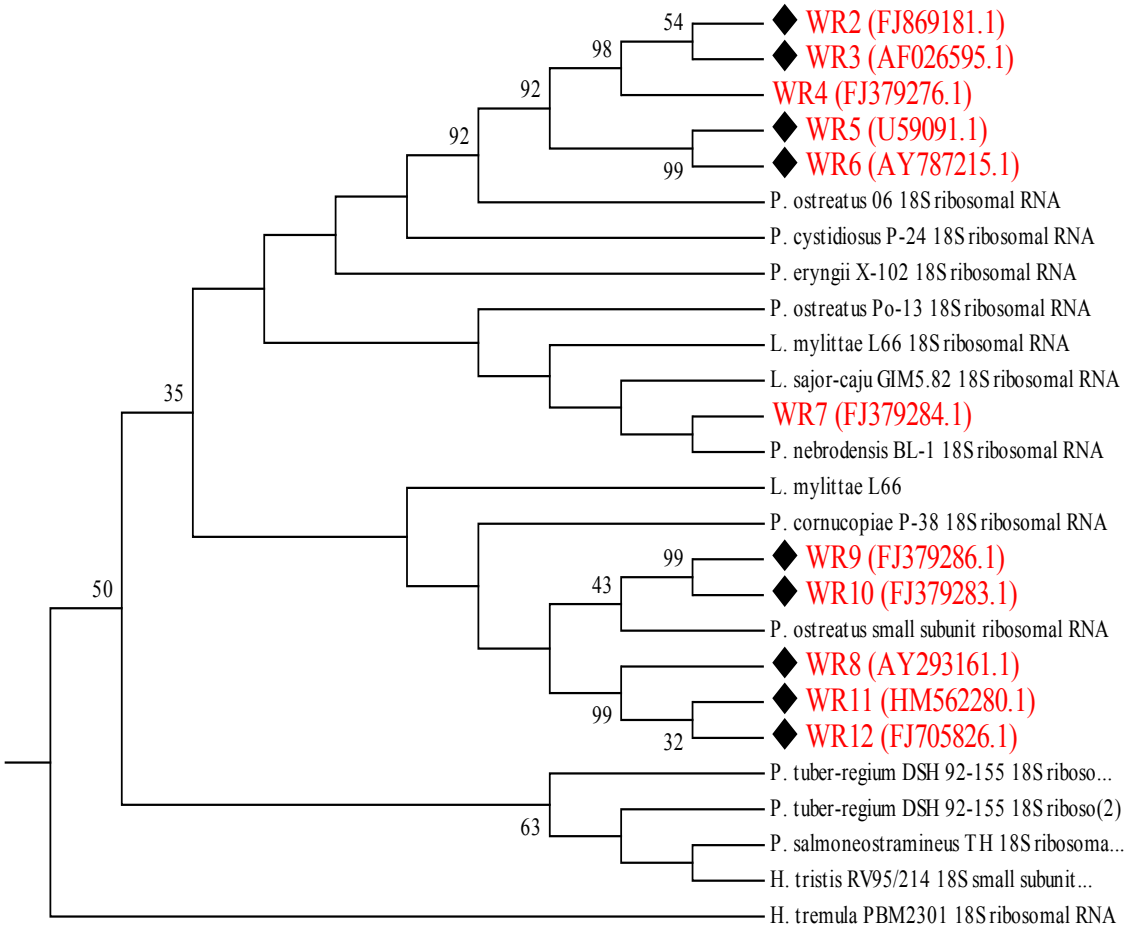

Fig. 13: Phylogenetic sub tree 1 showing position of isolate WR2, WR4 and WR9. The scale bar indicates approximately 1% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 1000 bootstrap replicates. The 18S rRNA gene sequence of *H. tremula* was used as an out group.

The phylogenetic position of isolate WR1 indicates that the isolate clusters with the genus *Coriolopsis* while WR15 indicates clustering with uncultured. This clustering pattern was supported by high bootstrap values of 99 % for WR1. The isolate WR15 could be a new species with a bootstrap of 94% (Figure 14). The isolate WR1 had sequence similarity of 99 % to *Fomes fomentarius, pycnoporus sp among others* (Appendix 1). Isolate WR15 had sequence similarity of 94% to *Uncultured stramenophile* among others (Appendix 1)

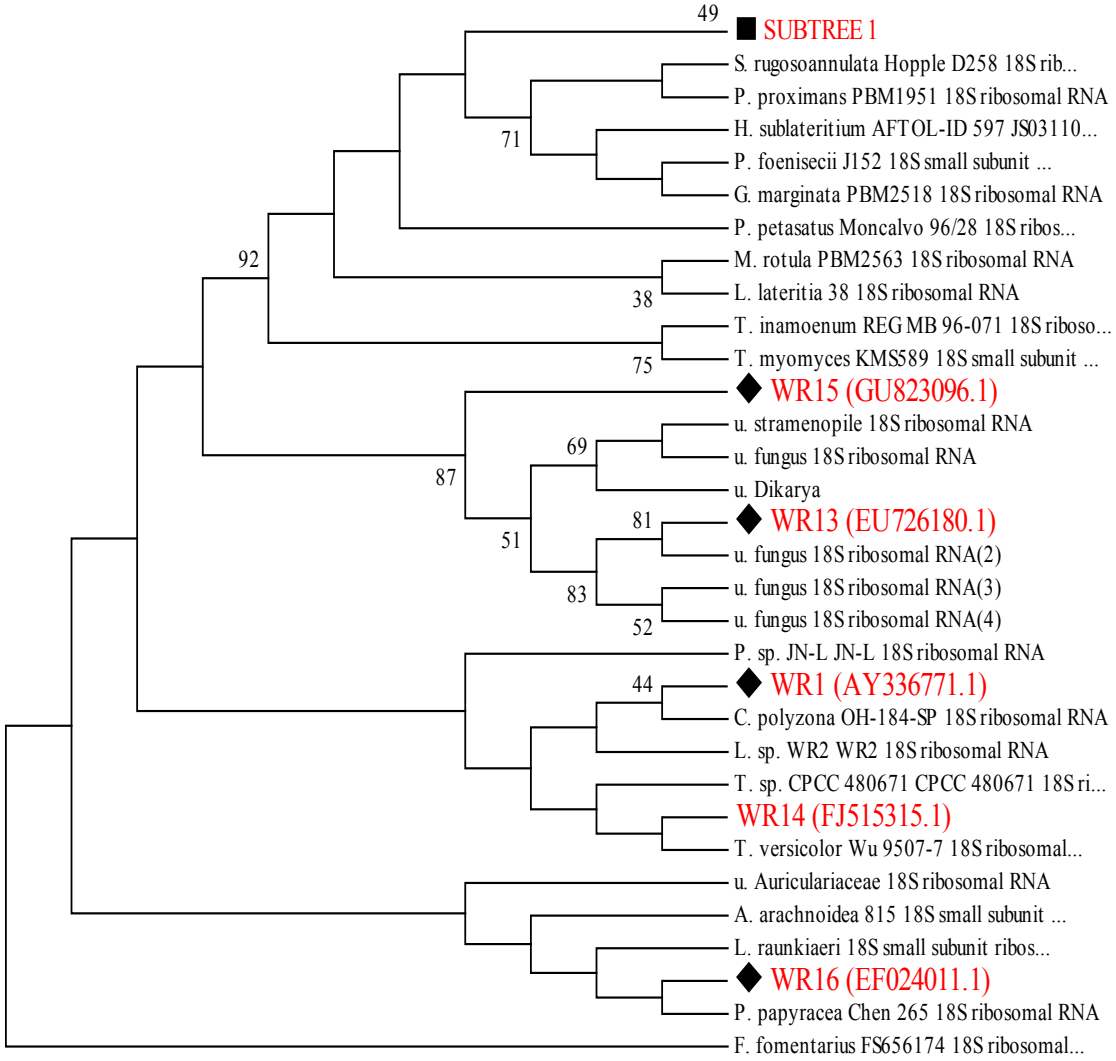

Fig. 14: Phylogenetic sub tree 2 showing position of isolate WR1 and WR15. The scale bar indicates approximately 1% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 1000 bootstrap replicates. The 18S rRNA gene sequence of *F. fomentarius* was used as an out group.

CONCLUSION

In conclusion, the findings of this study and other related studies on biodegradation suggest that white rot fungi have potential for use in the remediation of soils contaminated with hazardous compounds, including diazinon and methomyl. The results from field studies, however basic, are extremely valuable for directing future research and for demonstrating complications that arise when bioremediation is applied at a large scale. The base of knowledge on bioremediation

capabilities of white rot fungi is growing rapidly from laboratory studies so the next step is to utilize this pool of information in an exploratory way in the field. Considering the serious consequences on human and ecosystem health that some of the above- mentioned contaminants create, the sooner we find a set of preliminary sustainable solutions, the better. White rot fungi may play a large role in this search, providing an environmentally- friendly, economical approach that we are really just beginning to understand. However, before the use of these fungi can be considered a viable alternative, the nature, toxicity, and stability of the soil-bound products must be elucidated under a variety of conditions.

Research should be concentrated to develop economical but effective microbial processes for treatment of industrial effluents containing these pesticides and taking them to field. More understanding is required to improve the accurate prediction of the environmental fate of pesticides. In order to reduce the problem of enhanced degradation of pesticides in soil, the rotation of crops and of pesticides is recommended. These approaches may lead to the reduced use of pesticides.

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APPENDICES

Apendix 1: 18s rRNA gene sequence similarity of white rot fungal isolates to other close relatives

Sequence code	Bp	Accession	Next neighbour in blast	% similarity
WR2	549	FJ869181.1	Pleurotus cornucopiae strain P-38	99%
		FJ379278.1	Lentinus sajor-caju strain GIM5.82	99%
		AY657010.1	Chlorophyllum agaricoides isolate AFTOL-ID 440	98%
WR9	495	FJ379286.1	Pleurotus eryngii strain X-102	96%
		U23544.1	Pleurotus ostreatus SSU	96%
		DQ851579.1	Phyllotopsis nidulans strain RV96/1	95%
WR10	545	FJ379283.1	Pleurotus cystidiosus strain P-24	98%
		DQ097341.1	Henningsomyces sp. FP-105017-sp	97%
		EU157728.1	Laccocephalum mylittae strain L66	98%
WR4	546	FJ379276.1	Pleurotus nebrodensis strain BL-1	99%
		AY944218.3	Laccocephalum mylittae strain L66	99%
		GU187659.1	Pseudoclitocybe cyathiformis JFA 12811	98%
WR3	545	AF026595.1	Pleurotus tuberregium	99%
		AF026634.1	Pluteus petasatus	98%
		FJ379285.1	Pleurotus salmoneostramineus strain TH	99%
WR8	549	AY293161.1	Tricholoma inamoenum	98%
		DQ367422.1	Tricholoma myomyces strain KMS589	98%
		DQ440645.1	Hohenbuehelia tremula isolate AFTOL-ID 1503	99%
WR7	403	HM562262.1	Pluteus albostipitatus voucher AJ154	92%
		DQ851578.1	Panaeolus foenisecii strain J152	94%
		DQ851573.1	Hohenbuehelia tristis strain RV95/214	95%
		FJ379284.1	Pleurotus ostreatus strain Po-13	96%
WR11	549	HM562280.1	Volvariella taylorii voucher AJ54	98%
		AF026635.1	Stropharia rugosoannulata	98%
		AF026633.1	Cortinarius iodes	98%
WR12	547	DQ113912.2	Marasmius rotula isolate AFTOL-ID 1505	98%
		DQ440635.1	Galerina marginata isolate AFTOL-ID 465	98%
		FJ705826.1	Pleurotus ostreatus isolate 06	99%
WR5	544	U59091.1	Pleurotus tuberregium	99%
		HM562281.1	Volvariella lepiotospora voucher AJ155	98%
		AY752970.1	Phaeomarasmius proximans isolate AFTOL-ID 979	98%
WR15	534	GU823096.1	Uncultured stramenopile clone A93F14RM2A10	94%
		AB534490.1	Uncultured fungus	94%
		HQ191287.1	Uncultured Dikarya	94%
WR6	542	AY787215.1	Hypholoma sublateritium isolate AFTOL-ID	98%
		HM347336.1	Lentinula lateritia isolate 38	97%
		HM562278.1	Volvariella caesiotincta voucher MA54717	98%

Continued…

Apendix 1 (cont.)

Sequence code	Bp	Accession	Next neighbour in blast	% similarity
WR1	557	AY336771.1	Coriolopsis polyzona strain OH-184-SP	99%
		HM584804.1	Fomes fomentarius isolate FS656174	99%
		GU182936.1	Pycnoporus sp. JN-L	98%
WR13	547	EU726180.1	Uncultured fungus clone DS_E12F	99%
		EU173089.1	Uncultured fungus clone G912P34FL4.T0	99%
		EU175637.1	Uncultured fungus clone G913P35FH16.T0	99%
WR14	548	AY336754.1	Pachykytospora papyracea strain Chen 265	99%
		GQ899200.1	Lentinus sp. WR2	99%
		FJ515315.1	Trametes sp. CPCC 480671	99%
WR16	547	EF024011.1	Uncultured Auriculariaceae clone	88%
		GU187640.1	Leptosporomyces raunkiaeri CFMR:HHB-7628	88%
		AY336751.1	Trametes versicolor strain Wu 9507-7	87%
		AF518572.1	Athelia arachnoidea	88%

The accession numbers are from the NCBI database