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

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Abstract
White rot fungi are robust organisms and are generally more tolerant to high concentrations of polluting chemicals than bacteria, they therefore present a powerful prospective tool in bioremediation. In this study, the potential for biodegradation of methomyl and diazinon by white rot fungi through enrichment and isolation of methomyl and diazinon biodegraders from horticultural soils was done. Five white rot fungal isolates WR1, WR2, WR4, WR9 and WR15 were cultured in a medium containing methomyl and diazinon 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. Using Gilson HPLC system with acetonitrile (75% sample: 25% acetonitrile) as the mobile phases. The biodegradation of methomyl and diazinon overtime using fungal isolate mixtures, took 59 days while for individual isolates, it took a maximum of 100 days to biodegrade the pesticides. These proofs that fungal mixtures in soil fasten the rate of biodegradation of pollutants compared to individual isolates. The pesticide methomyl was eluted at 4.9 minutes while the methomyl metabolite was eluted at 4.1 minutes. Diazinon was eluted at 11 minutes while the diazinon metabolites; diazoxon and oxypyrimidine were eluted at 2.3 and 2.6 minutes. The HPLC method used enabled the separation and quantification of the pesticides in an HPLC run-time of 15 min. Results indicated that after 100 days all the isolates managed to biodegrade the respective pesticides. The rate of mineralization or disappearance of a pesticide was proportional to the concentration of the pesticide. White rot fungi are advantageous over bacterial systems since these fungi can grow rapidly when supply of nutrients is low/limited.

Key words: White rot fungi, methomyl, diazinon, metabolite and biodegradation

1.0 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 (Reddy and Mathew, 2001). 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 (Reddy and Mathew, 2001). 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. One growing mechanism of decontamination that may fit these requirements is bioremediation. Utilizing microorganisms to degrade toxic organopollutants is an efficient, economical approach that has been successful in laboratory studies. Interest in bio-remediation as an alternative approach to clean-up has increased. Research on biodegradation has demonstrated the potential of white-rot fungi to degrade PAH (Boopathy, 2000).

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. Degradation products of diazinon include diazoxon and oxypyrimidine (Desaint *et al*., 2000). Oxypyrimidine is the main soil and water degradate of diazinon. Diazinon can be converted to diazoxon in the atmosphere via ultraviolet (Sethunathan, 1972). 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's influenced by the pH conditions in the soil and the soil type (Sethunathan, 1972). In agricultural soils, methomyl is rapidly mineralized to carbon dioxide. No other degradation compound is observed in soil in significant amounts (Strathman *et al*., 2001). Methomyl oxime is a minor transient degradation product observed at a maximum of 2.9% of applied active ingredient. Methomyl does not leave any significant residues in soil after application (Tomlin, 2003).

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* (Hestbjerg *et al*, 2003). These fungi cannot use lignin as a source of energy, however, and instead require substrates such as cellulose or other carbon sources. Thus, carbon sources such as corncobs, straw, and sawdust can be easily used to enhance degradation rates by these organisms at polluted sites. Also, the branching, filamentous mode of fungal growth allows for more efficient colonization and exploration of contaminated soil. 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 (Cajthaml *et al*, 2002; Mansur *et al*, 2003; Pointing, 2003, Veignie, 2004). The three main LMEs are lignin peroxidase, Mn-dependent peroxidase, and laccase. All three of these enzyme groups are stimulated by nutrient limitation (Mansur *et al*, 2003; Aust *et al,* 2004).

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. Overall, most of these physical-chemical cleaning technologies are expensive but 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 (Kearney, 1998; Nerud *et al.*, 2003) because the contaminated soil has to be excavated at a site and moved to a storage area where it can be processed. 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 (Zhang and Quiao, 2002). For this reason several biological techniques involving biodegradation of organic compounds by microorganisms like bacteria and fungi (white rot fungi) have been developed (Schoefs *et al.*, 2004).

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.

The full potential of biodegradation by white-rot fungi has not been fully investigated for field soils. The objectives of this work were to evaluate the ability of selected white-rot fungi (WR1, WR2, WR4, WR9 and WR15) to degrade Methomyl and Diazinon. All possible combinations of these white rot fungi were also investigated to determine whether the use of fungal consortia could promote enhanced degradation. Experiments were designed so that the results obtained reflected not only differences in degradation potential, but also those related to speed and extent of fungal growth on the organic substrate, tolerance to high levels of methomyl and diazinon. This paper will review the research thus far on the potential use of microorganisms (especially white-rot fungi) in degrading some of the top pollutants (methomyl and diazinon).

2.0 Materials and Methods

2.1 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.

2.2 Soil Particle Washing and Plating

Soil cores of 2.5 cm diameter were taken to 5 cm depth after the litter layer was removed. Sampling was done using stratified random sampling method from two regions in Kenya; Rift-valley region and Mt. Kenya region. 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. 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 (Bingle and paul, 1986). 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 (Bingle and paul, 1986).

2.3 Composition of the Media

Soil particle-washing technique (Bååth, 1988, Bills and Polishook, 1994, Bissett and Widden, 1972) was used to remove spores of ascomycetous and zygomycetous molds, and plated the washed particles on a medium made selective for basidiomycetes by the incorporation of benomyl as described by Bååth (1988) (Table 1). In addition to benomyl, the medium contained lignin, to encourage selection of ligninolytic fungi, and guaiacol, which acts as a colorimetric indicator of the lignin-modifying enzymes laccase or peroxidases (Arora and Sandhu, 1985). All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. (Greg *et al*., 1996 and Domsch *et al*., 1980).

Table 1: Composition of media for isolation of white rot fungi as described by Bååth, 1988

Ingredients	*g/l*	*Ml/l*	*Concentration*	*Mg/l*
KH_2PO4	0.5			
$MgSO_4.7H_2O$	0.2			
NH_4NO_3	0.1			
KCl	0.1			
$FeSO_4.7H_2O$	0.02			
$Ca(NO3)_2.4H_2O$	0.05			
Malt extract	2			
Agar	15			
KOH		5	1M	
Guaiacol		0.4		
Indulin AT	1			
Dioxane		10		
Chloramphenicol	0.25			
Benomyl				4
Acetone-70% ethanol		2		

2.4 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) (Summerbell, 1985).

2.4.1 Minimal Mineral Media for Methomyl and Diazinon Liquid Cultures

Mineral medium MMN (mineral medium without nitrogen and carbon) was derived from mineral medium MMO (Stainer *et al.,* 1966) by elimination of all nitrogen. MMN medium contained 1,419.6 mg of Na_2HPO_4, 1,360.9 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 (Fluka AG Chemische Fabrik, Buchs, Switzerland).

2.4.2 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^oC 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.

2.5 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 30^0Cwith 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. At periodic intervals 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 (George *et al*., 2005).

2.6 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). These compounds were identified by their retention times and peaks corresponding to reference standards.

3.0 Results

3.1 Morphological Characterization of White Rot Fungal Isolates

Figure 1a 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 figure 1b shows the front side of the same plate which is characterized by the growth of mycelia. Figure 1c shows a plate with high concentration of lignin hence the darkening color.

(a)

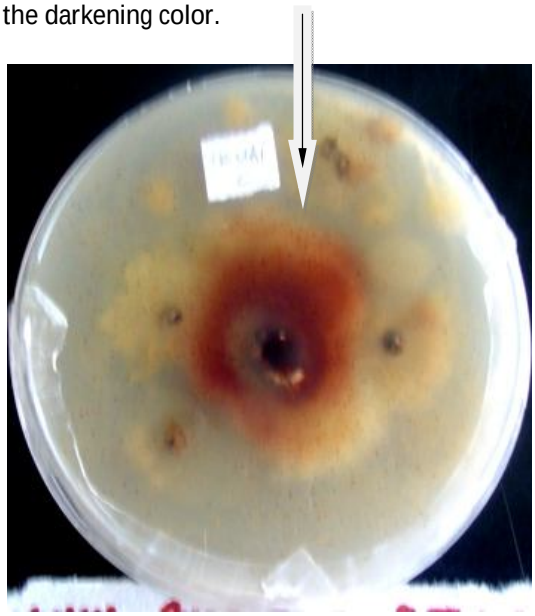

Figure 1a: Back side of plate showing isolates growing on Lignin Guaiacol Benomyl agar with low concentration of lignin

(b)

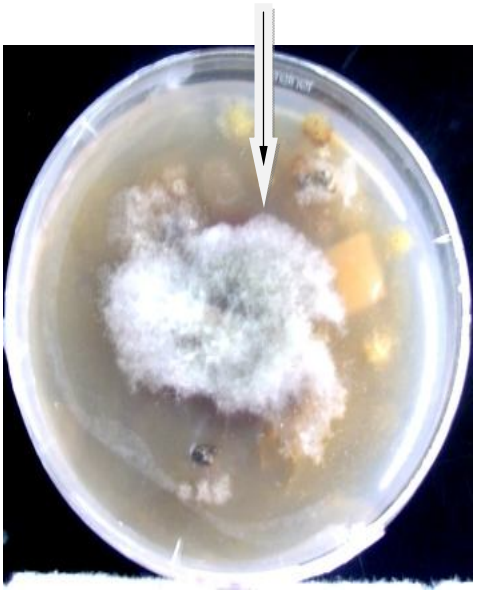

Figure 1b: Front side of plate showing isolates growing on Lignin Guaiacol Benomyl agar

(c)

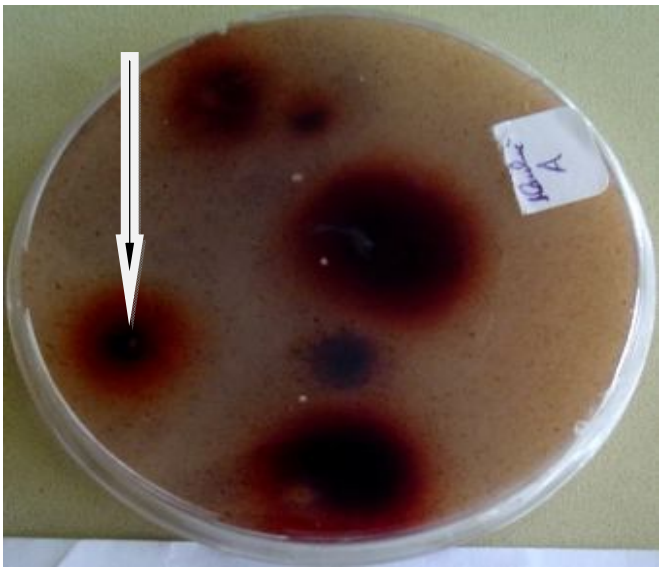

Figure 1c: Back side of plate showing isolates growing on Lignin Guaiacol Benomyl agar with high concentration of lignin

Figure 1 a, b and c. 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.

3.2 Microscopic Characterization of White Rot Fungal Isolates

Figure 2 shows clamp connections (arrows) as seen under the microscope at x100 objective lens.

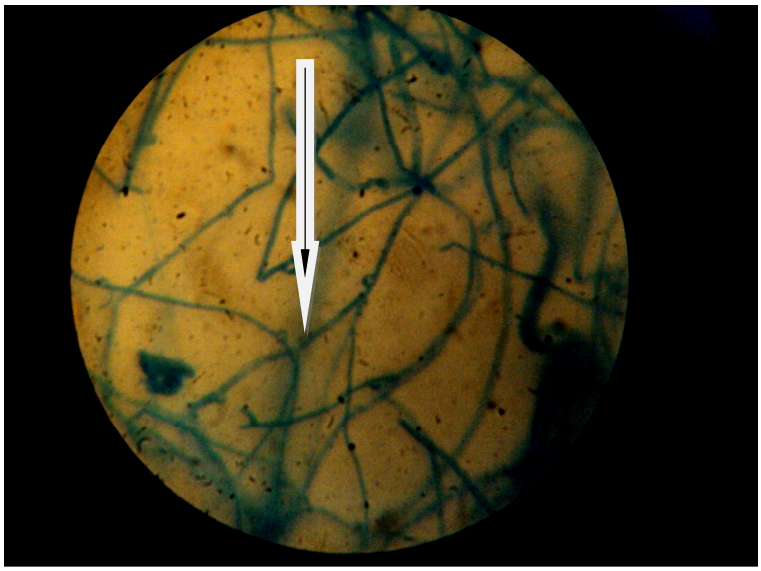

Figure 2: Isolate showing clamp connections (arrows) as seen under the microscope at x100 objective lense

Figure 3 below shows fruiting body as seen under the microscope at x100 objective lense.

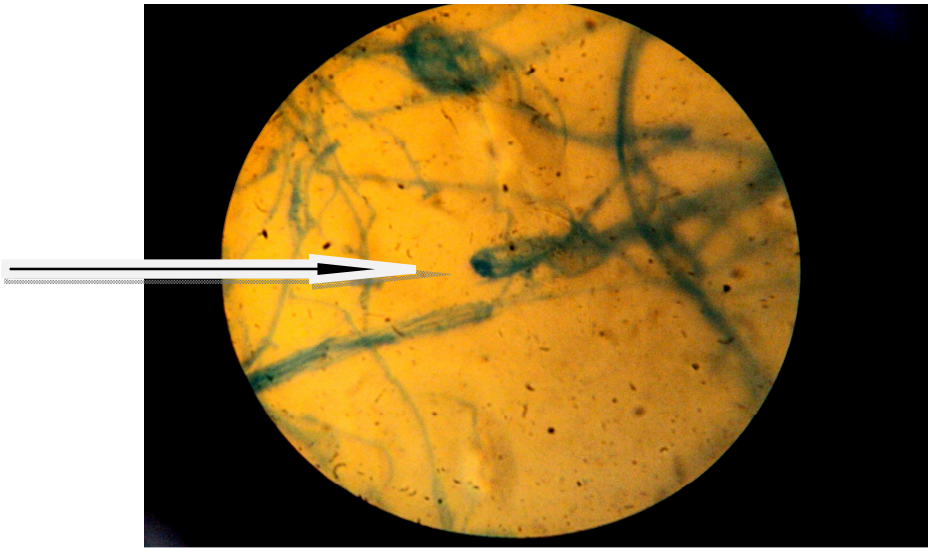

Figure 3: White rot fungi isolate showing fruiting body (arrow) as seen under x100 objective lense

Figure 3 showing white rot fungi isolate fruiting body (arrow) as seen under the microscope at x100 objective lense.

3.3 Biodegradation Profiles of Methomyl by White Rot Fungi

Figure 4a and b**.** Shows profiles of mineral salts (0 days) and methomyl pesticide (10 days) after a hplc run time of 10 minutes respectively.

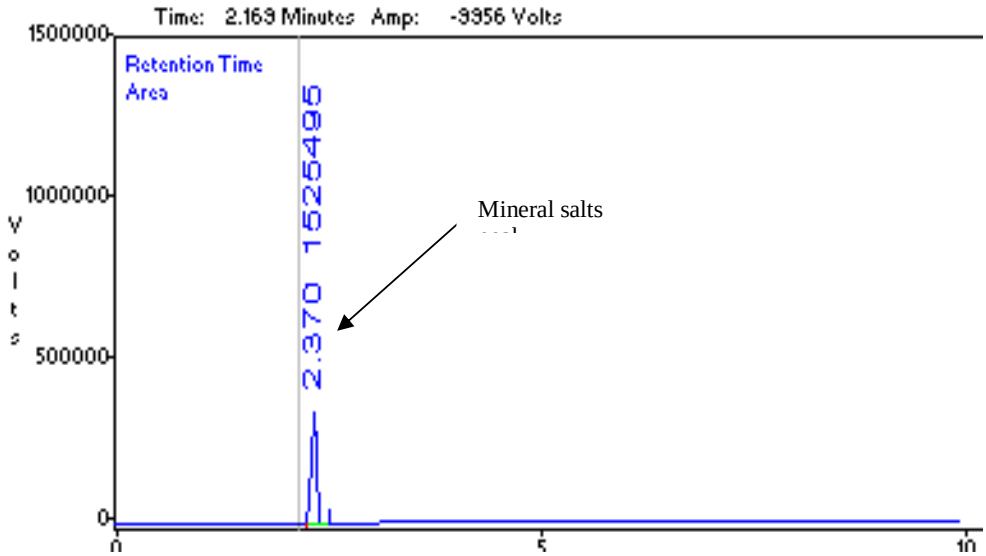

Figure 4a: Profile of Minimal mineral salt medium run in the Hplc at a retention time of 2.3 minutes (1525495) at a flow rate of 1ml/min for a run time of 10 minutes after 0 days

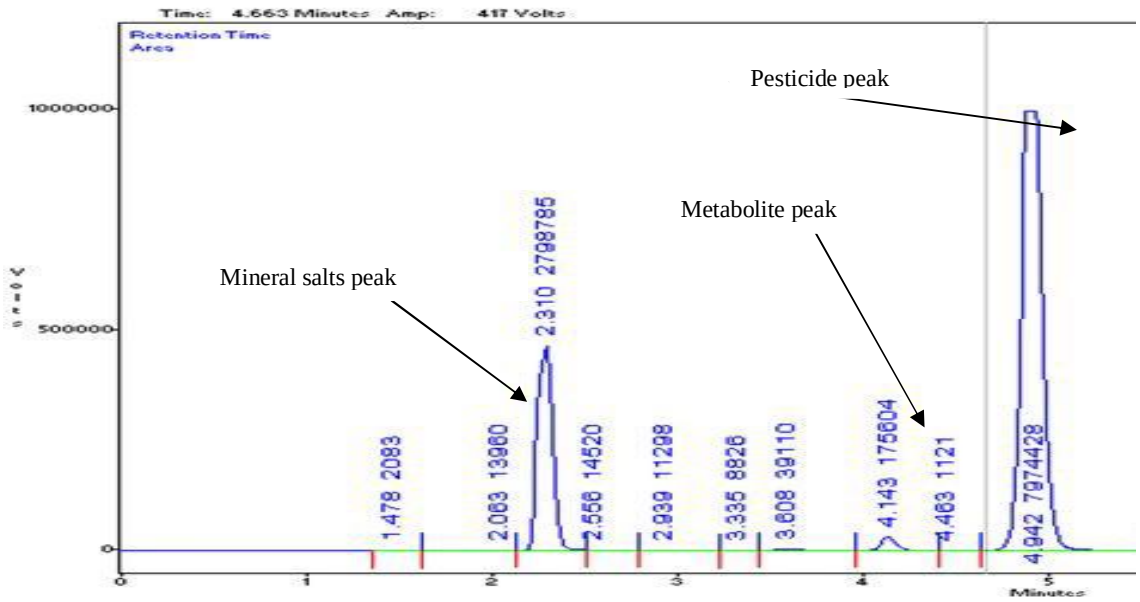

Figure 4b: Methomyl profile showing mineral salt peak at retention time of 2.3 minutes (2798785), metabolite peak at retention time 4.1 minutes (175604) and pesticide peak with a retention time of 4.9 minutes (7974428) after a hplc runtime of 10 minutes for 10 day old WR2 culture of white rot fungi

Figure 4c. Shows biodegradation profiles of a mixture of white rot fungi isolate WR2 and WR9 in methomyl pesticide (10 days old) after a hplc run time of 10 minutes.

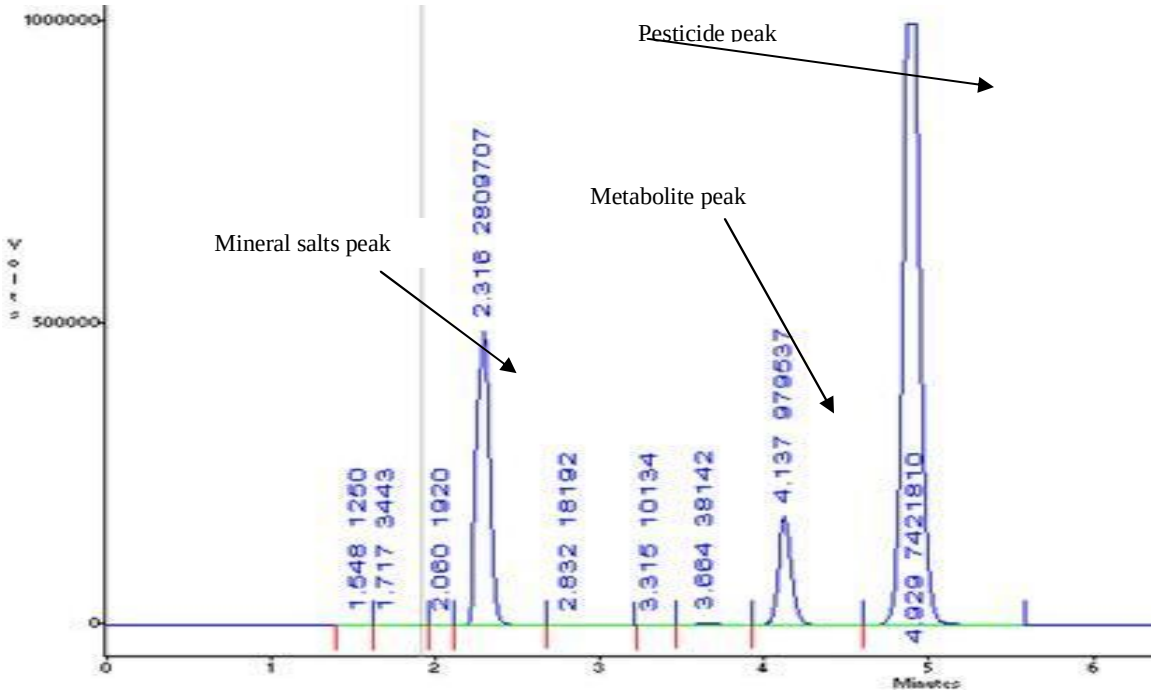

Figure 4c: Methomyl profile showing mineral salt peak at retention time of 2.3 minutes (2809707), metabolite peak at retention time 4.1 minutes (979537) and pesticide peak with a retention time of 4.9 minutes (7421810) after a hplc runtime of 10 minutes for 10 day old WR1 & WR9 cultures of white rot fungi

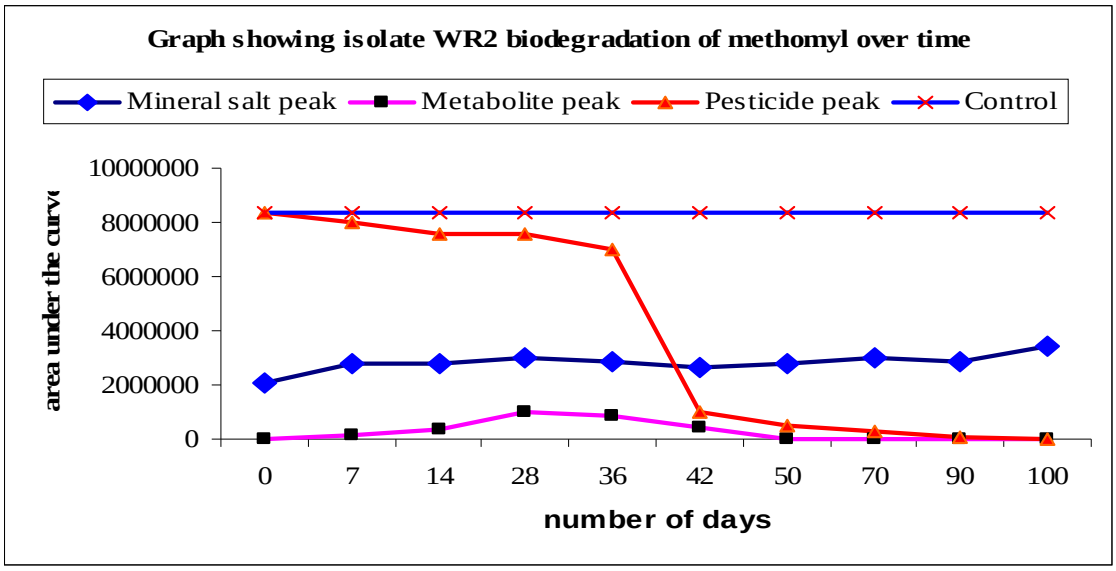

Figure 5a: Methomyl biodegradation profile of isolate WR2 over a period of 100 days

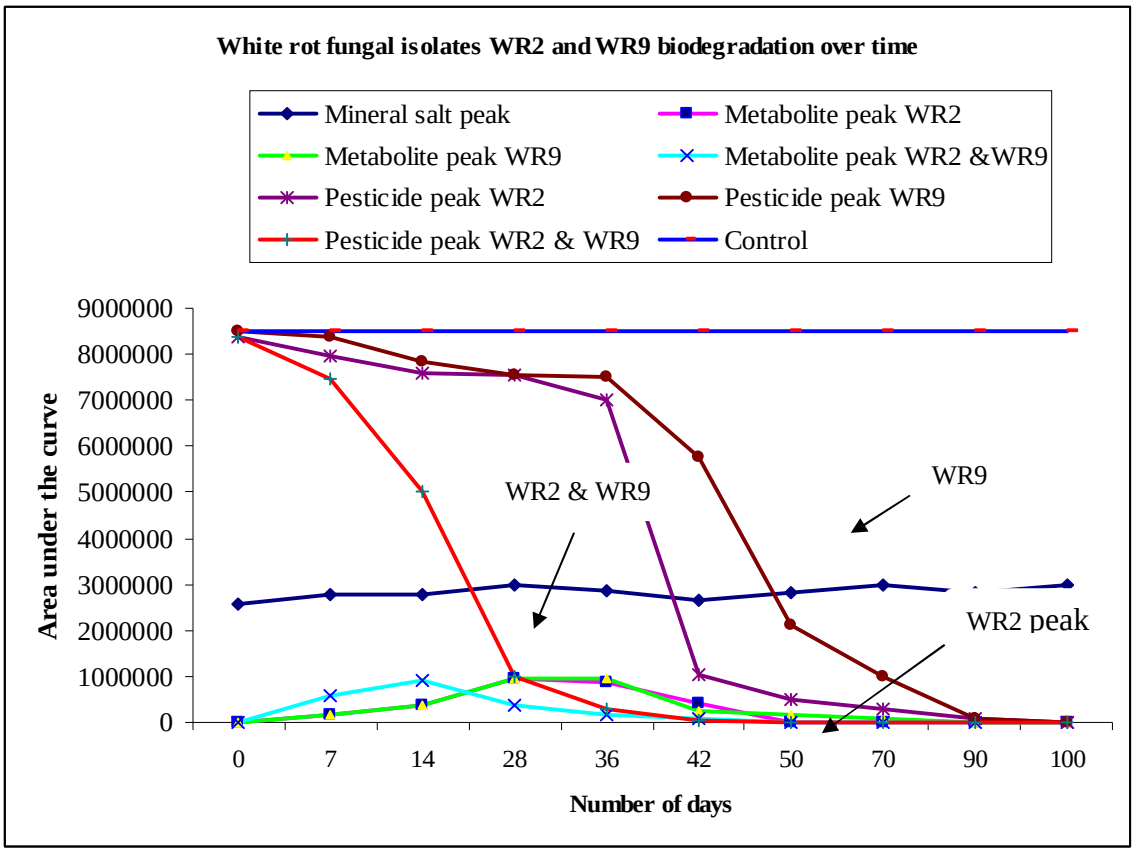

Figure 5b: Methomyl biodegradation profile of a mixture of isolate WR2 & WR9 over a period of time

3

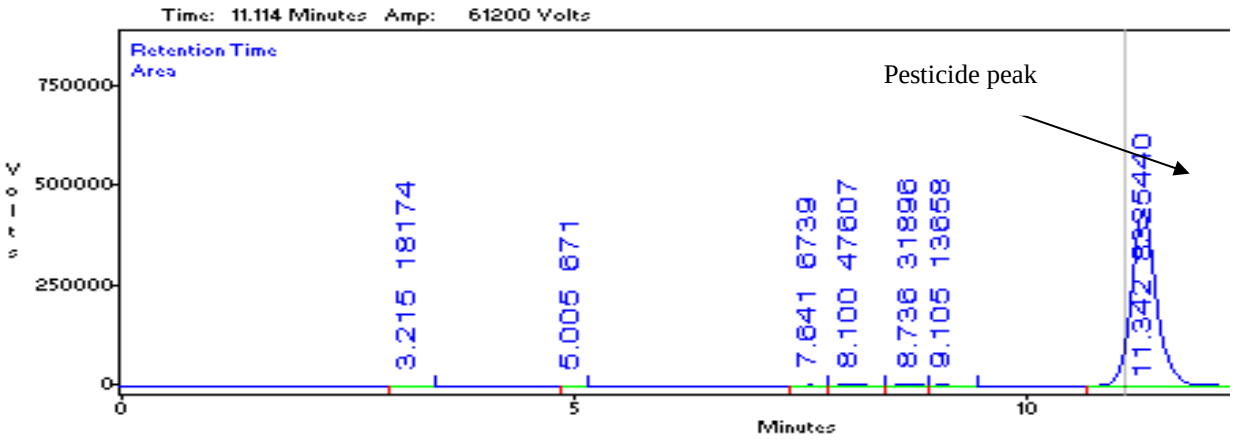

Figure 6a: Profile showing peak of diazinon pesticide with time retention of 11 minutes (8325440)

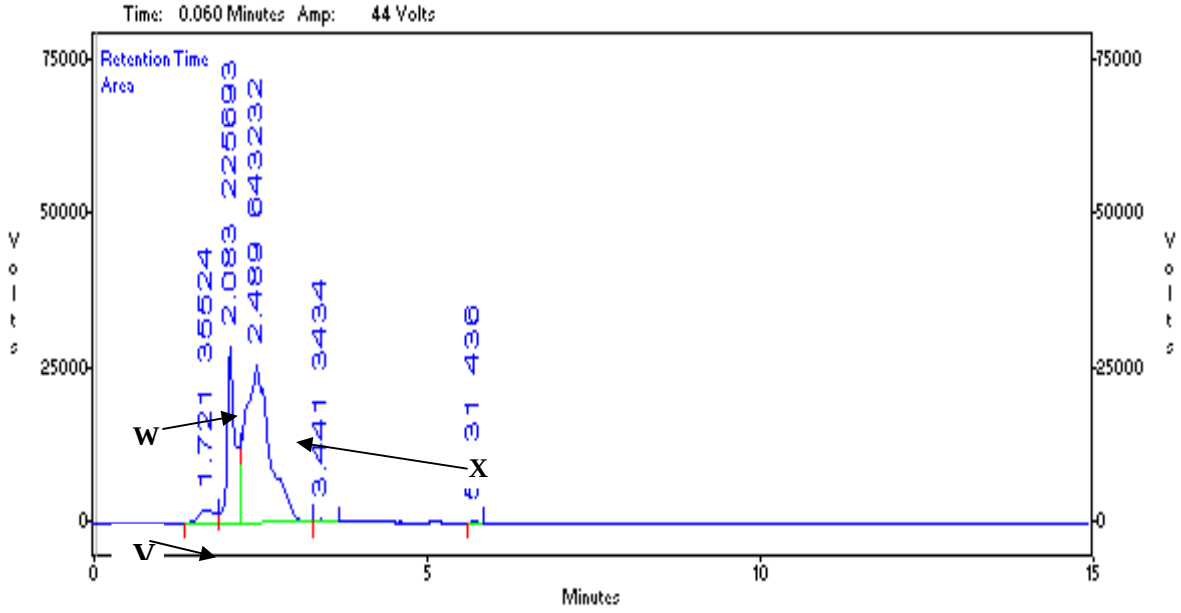

Figure 6b: Diazinon mineral salt peak V, W & X at retention time 1.7 minutes (35524), 2.0 minutes (225693) and 2.4 minutes (643232) respectively

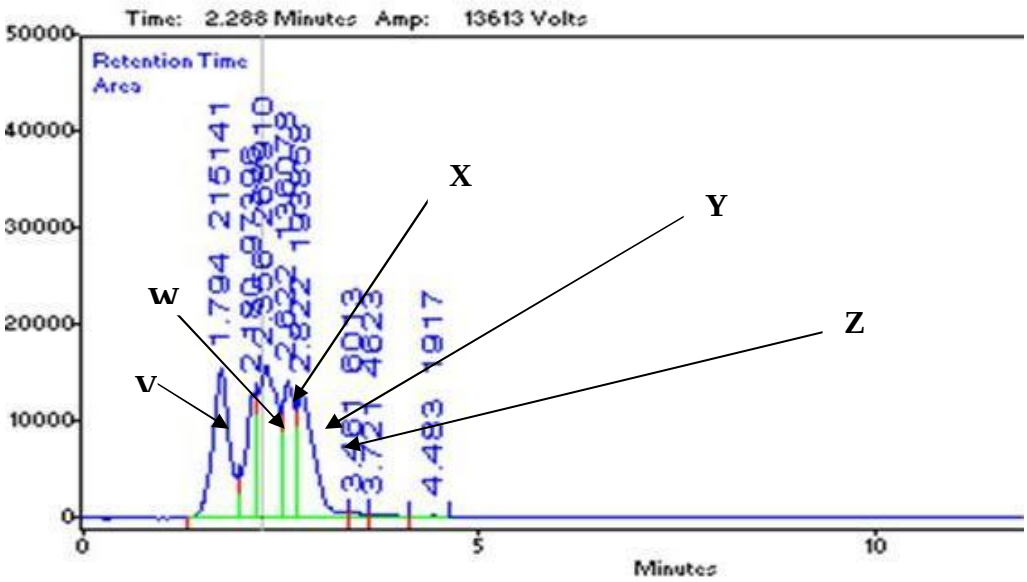

Figure 6c: Profile showing complete biodegradation of the pesticide diazinon by white rot fungi isolate WR15 after 80 days. The mineral salt peaks are V at retention time 1.7 minutes (215141), W retention time 2.1minutes (97396), X retention time 2.3minutes (268910) while the metabolite peaks are; Y retention time 2.6minutes (136078) and Z retention time 2.8 minutes (193858)

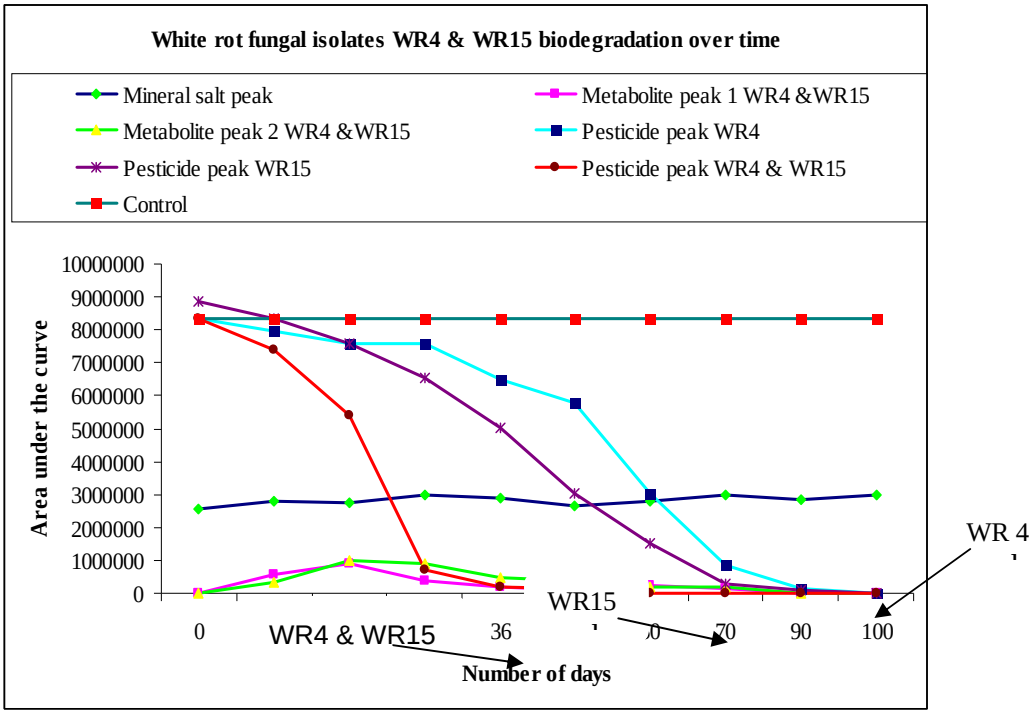

Figure 6d: Diazinon biodegradation profile of white rot fungi isolates WR4 &WR15 over a period of 57 days

4.0 Discussion

In these study, two major findings in biodegradation of contaminated soils namely; the ability of white-rot fungi in Methomyl and 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 (2003) 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 (Boopathy, 2000), hence possible use of white rot fungi may be an important factor in this solution.

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 (Magan and Lacey, 1984; Marin *et al*., 1998a; Marin *et al*., 1998b). Therefore, better growth could help the introduced fungi to overcome competition from indigenous soil microorganisms (Singleton, 2001). Since mixtures of microorganisms exist in soil (Aust, 2003), 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 (Reddy and Mathew, 2001).

The results in Figure 4 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 (2001), 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 (2001) agrees with our results which show that the metabolite peak at 4.1 minutes disappeared over time before degradation of methomyl was achieved. Veignie (2004) 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 5a & 6d where degradation took 100 days to be complete for a single isolate. However, when the isolates were mixed (figure 5b and 6d), degradation took half (50-57 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.

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 6d and 6c. This agrees with the work of Sethunathan (1972) 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 (1971), 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 (Suet, 1971).

We conclude that 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.

5.0 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. 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. Selected white rot fungi could prove valuable in on-farm pesticide bioremediation systems.

6.0 Recommendations

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. Selected microbial cultures are now available to set up industrial processes for decontamination of effluents, agricultural wastes and dump sites. Such an approach is likely to be efficient and cost-effective for many problems. Genetically modified microorganisms can provide improved activity which should prove useful in large-scale applications of microbial degradation to environmental problems. However, it is essential that such microorganisms are thoroughly evaluated for safety before release into the environment.

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