Isolation and Characterization of Linuron Degrading Bacteria from Soils under Horticultural Production in Kenya

Philip Miriti[1,*], Gabriel Magoma.[2], Hamadi I. Boga[3], Aggrey Nyende B[4]

[1]Institute of Biotechnology Research Jomo Kenyatta University of agriculture and Technology P.O Box 62000 (00200) Nairobi, Kenya
[2]Pan African University Institute of Basic Science Technology And Innovation Jomo Kenyatta University of agriculture and Technology – AICAD Block C, Room 101 P. O Box 62000 00200 Nairobi, Kenya
[3]Hamandi Iddi, Jomo Kenyatta University of agriculture and Technology P.O Box 62000 (00200) Nairobi, Kenya
[4]Institute of Biotechnology Research Jomo Kenyatta University of agriculture and Technology P.O Box 62000 (00200) Nairobi, Kenya
*Corresponding author: philipmwenda2012@gmail.com

Abstract Pesticides use has been one of the major factors in improving productivity in agricultural enterprises in Kenya. Phenylurea herbicides are one of the main categories of crop protection products that kill weeds and other plants that grow where they are unwanted. In recent years researchers have been paying greater attention to this family of herbicides because of their high biotoxicity and possible carcinogenic properties and lengthy periods of time for their removal from the environment. However, systematic studies on microbial removal of these pesticide residues are scarce in developing countries including Kenya. A survey in four horticultural regions of central and rift-valley Kenya showed that out of the twenty two (22) formulations of pesticides used linuron had the highest frequency of application at 45.9%. Through enrichment and isolation a total of six isolates were obtained from linuron contaminated soils. Degradation kinetics of the pesticides residues was monitored by High pressure liquid chromatography (HPLC). Level of degradation was scored by the data fit with a linear regression by the line y =49508x with an R2 value of 0.9831. The corroboration of various degradation intermediate metabolites was aided by GC-MS machine. The identified metabolites from linuron were 3, 4 dichloroanilline, 3,-chloroanalline, 4-chloroanalline N, O dimethylhydroxylamine, and N-methyl-N-methoxy carbamic acid methyl ester. Isolates designated LoG-8A, Lwa-2A, Lsh-6B, LJk-5C, Lla-1A and Lwa-2C showed the ability to degrade 70.7- 98.9% of 50mgl^{-1} of linuron within 84 days. Morphological, biochemical and 16S rRNA sequence analysis of these isolates indentified them with the genus *Pseudomonas, Stenotrophomanas, Burkholderia, Lysinibacillus, Arthrobacter* and *Flavobacteria.* The study demonstrated that soils from horticultural farms in Kenya harbor biodegrades for linuron. Further work can show whether these microbes can be used for the development of bioremediation strategy.

Keywords: *pesticides, biotoxicity, bioremediation, Linuron and contamination*

1. Introduction

The horticulture industry a sub sector in agriculture is one of the fastest growing in the Kenya and the second largest foreign exchange earner after tea in Kenya [21]. Apparently pests and diseases are responsible for 30-40% loss in agricultural produce in the tropics [5]. The most conventional and common way of pest and disease control is through the use of pesticides. Among these substances, the family of phenylurea herbicides has received particular attention in recent years because of its high biotoxicity and possible carcinogenic properties. Moreover, these compounds require several weeks to months for their removal from the environment [4]. Unfortunately, these compounds pose inherent toxicities that endanger the health of the farm operator, consumer and the environment [5]. Many of these compounds have been observed to persist in the environment, biomagnify when passing through food chains and bioaccumulate in tissues [27]. However, the horticulture industry in Kenya is the major consumer of pesticides. In 2005 approximately 7047 metric tonnes of the pesticides were imported into Kenya for control of plant pest and diseases [25]. Worldwide, weeds cause more yield loss and add more to farmers' production costs than insect pests, crop pathogens, root-feeding nematodes, or warm-blooded pests (rodents, birds, deer, and other large grazers). Moreover, Weeds compete with crop for space, light, moisture and nutrients, and reduce the crop yield by 17 to 25% [22]. Phenylureas, which are commercially available

for more than 50 years, represent one of the most widely used classes of herbicides to control germinating and emerging grasses and broad-leafed weeds in field and fruit crops [25]. In recent years researchers are paying greater attention to this family of herbicides because of their high biotoxicity and possible carcinogenic properties and they require several weeks to months for their removal from the environment [5].

Most of the herbicide load enters the environment as diffuse contamination from agricultural sources following normal spraying practices. Several factors such as the dynamics of the water flow, sorption to solid phases, plant uptake, microbial and (photo) chemical degradation, and dissolved and colloidal phase migration affect herbicide dispersion under natural conditions [13]. Phenylureas are prone to photochemical degradation; however, the process can lead to photoproducts such demethylated or formylated derivatives which have a higher toxicity than that of the mother compound [1]. Different phenylurea herbicides have been detected as pollutants of groundwater and surface water and in some cases residues have been detected three years after the final application [16-31].

Environmental effects of Linuron mainly concern aquatic ecosystems since linuron has been shown to be highly toxic to aquatic plants and invertebrates [13]. The European Union has included Linuron in list II of the Dangerous Substances Directive (76/464/EEC) to encourage member states to reduce the use of this compound [36]. Also potential metabolites formed during abiotic and/or biotic degradation of phenylureas (for example chlorinated anilines) are included in this list as they may cause long term adverse effects in the aquatic environment. [35] has demonstrated that both 4-isopropylaniline (4-IA) (derived from isoproturon) and 3, 4-DCA (derived from linuron) displayed a much higher toxicity than their mother compounds in a Microtox® assay.

The slow natural biodegradation rate of phenylurea herbicides observed in both field and laboratory studies and the detection of several of these herbicides and their metabolites as contaminants in groundwater and surface water bodies, including seawater has stimulated research to identify phenylurea-degrading microorganisms. The objective of this research was to isolate the bacterial strains, characterize their degradation potential and ascertain the degradation abilities in the utilization of linuron as the sole carbon source in liquid medium.

2. Materials and Methods

2.1. Chemicals

Analytical-grade linuron (Fluka CAT 36141; 99.5% purity) was purchased from Kobian limited (Nairobi, Kenya). Stock solutions of 100 mg L^{-1} were prepared in high pressure liquid chromatography HPLC-grade methanol (Sigma Aldrich UK). HPLC grade acetonitrile and water (Sigma Aldrich Germany) were used as mobile phase. Hexane (99.9%, Sigma Aldrich St. Louis, MO, USA) and $Na_2SO_4(s)$ ACS (Sigma Aldrich UK) were used for GC/MS analysis of degradation residues of the pesticides.

2.2. Soil Samples

The soil samples were taken from central and rift-valley regions of Kenya from eight farms under horticultural production that had been regularly treated with different urea herbicides. Eighteen soil samples (50 g each) were taken in different parts of each plot using a soil auger. The upper top soil (5 cm deep) mineral soil was collected cross-sectional within a given plot of ≈ 100 m^2 and placed in sterile polythene paper. Similar sampling was done 100 m away from the farms to act as controls. Samples from each plot were then mixed to obtain homogenous and placed in sterile plastic containers. Samples were then kept in flask containing ice and transferred to the laboratory within 12 hours and stored at 4°C.

2.3. Growth Media

The mineral salt medium (MSM) comprised of $Na_2HPO4.12H_2O_3$ 0.5 mg, KH_2PO_4 12 mg, $MgSO_4.7H_2O$ 2.5 mg, $CaCl_2.2H_2O$ 1.5 mg, $H_3BO_3.$ 62 µg, $Na_2MoO_4.2H_2O$ 242 µg, $MnCl_2.6H_2O$ 198 µg, $FeCl_3.6H_2O$ 54 µg, $CuSO_4.5H_2O$ 1.3 µg, and $ZnSO_4.7H_2O$ 1.4 µg dissolved in one liter of Milli-Q water. Each batch of medium was sterilized by autoclaving at 121°C. To prepare solid media, each chemical dissolved in Milli-Q water was added to each medium by filter sterilization. To prepare liquid media, each chemical dissolved in methanol was poured into heat-sterilized glass vials or bottles, and organic solvent was then removed by a filter-sterilized nitrogen stream. 5 ml of the liquid medium was then poured into the vial or bottle and allowed to stand overnight to ensure that the chemicals were sufficiently dissolved in the liquid

2.4. Enrichment and Isolation Procedure

2.5 g of sieved soil (<2 mm) in 25 mL of MSM with linuron (25 mg L^{-1}) was used as the only source of carbon. Incubation was done at 25 °C on a rotary shaker (1200 r.p.m.). Soil particles present in these cultures served as a solid support for sessile growth. Sterile controls of the soils were incubated simultaneously to monitor abiotic losses of linuron during the enrichment. Linuron concentrations was monitored via HPLC, and when the concentration of linuron in the medium was below the detection limit (0.5 mg L^{-1}) 50% of the volume of the cultured sample was picked after settlement of the soil particles by centrifuging at 1200 g for 5 min. The flasks were replaced with fresh medium containing 50 mg L^{-1} linuron. After three successive refreshments Linuron fed enrichment cultures without solid support were set up from these cultures. 250 µL of the cultures was transferred to 25 mL of MSM supplemented with 25 mg L^{-1} linuron. To make sure that all soil particles were removed, the cultures were transferred in the same way three times after linuron concentration had decreased below the detection limit. All linuron fed enrichment cultures were enriched for 12 weeks before plating.

2.5. Batch Degradation Experiments

Degradation of Linuron by the enriched cultures was tested by inoculating 250 µL of the cultures in 25 mL of MSM supplemented with 50 mg L^{-1} linuron.

Concentrations in the cultures and controls were monitored with HPLC during incubation. Tests were performed in triplicate, and uninoculated vials containing the same media were incubated simultaneously to determine abiotic losses. Utilization of linuron as a carbon source was determined by following the change in $OD_{600 nm}$ on a Spectrophotometer (Pharmacia Biotech Novaspec II, 1996) in 8 mL of MSM supplemented with the herbicide. After each experiment, the cultures were plated on MSM agar medium to rule out possible contamination.

2.6. HPLC Analysis

Cultures (including sterile controls) were sampled and analyzed by reverse-phase HPLC ((Shimadzu HPLC class *VP* series) after centrifugation at 8500 *g* for 10 min. The HPLC system was equipped with a C-18 silica reverse phase guard column and a C-18 column, 250 x 4.6mm (Fisher Scientific, Fairlawn, N.J). UV detector set at 254 nm. The mobile phase consisted of acetonitrile and water (70/30) with a flow rate of 1 mL min^{-1} [9]. Identification and quantification were based on comparison to the retention times and peak areas of external standards of linuron.

2.7. GC/MS Analysis

Cultures that showed decrease in concentration in HPLC were further analyzed by GC-MS to identify the metabolites of degradation. Liquid samples (10 ml) were fulfilled to volume of 50 ml with deionized water and extracted (2x with) with 20 ml hexane on a rotary shaker for 1 h. Next, the extracts were dehydrated with anhydrous Na_2SO_4 and evaporated to dryness under a stream of N_2 at 45°C using a rotary evaporator (IKA, RV Basic, Janke and Kunkel-labortechnik, Germany) and subsequently diluted to a final volume of 10 ml with acetone and reserved for chromatographic analysis. 30 µl of the extracts were placed in auto sampler inserts before placing in the auto sampler vials and injected directly for GC- MS analysis. Library - MS searches using NIST/EPA/NIH MASS SPECTRAL LIBRARY (NIST 05) and NIST MASS SPECTRAL SEARCH PROGRAM Version 2.0d, chemco and Adams data base were used for characterization purposes in the GC-MS data system.

2.8. Characterization of Isolates

Bacterial strains preliminary characterization was performed using morphological and cultural characteristics as described by (Holt *et al.,* 1994). Morphological identification of the isolate was done under the dissecting and compound microscope to observe cell size, shape and arrangement characteristics after classical staining of bacteria. 3% (w/v) KOH [11] was used to determine gram characteristics of isolates. Simple, differential, and structural even if combined with cultivation and observation of bacterial isolates were not sufficient for the identification of the bacterial isolates [3]. Therefore further characterization was done through biochemical tests to evaluate the metabolic properties of isolates, which are unique for each species and the identities of the isolates confirmed through molecular characterization.

2.9. Polymerized Chain Reaction

Genomic DNA was extracted from bacterial cells grown on plates containing 10-fold diluted agar (2 mg L^{-1} linuron) as described previously [37]. All PCR reactions were performed in a Mastercycler from Eppendorf (Hamburg, Germany). 16S rRNA gene fragments of the pure isolates were amplified using eubacterial primers 27F and 1492R [24]. In both cases, the PCR mixture contained 1 µL of DNA extract, 5 µL of 10 × PCR buffer (Qiagen), 5 µL of 1% bovine serum albumin (BSA) (Amersham Biosciences), 4 µL of dNTPs (2.5 mM each) (Invitrogen), 0.25 µL of each primer (0.1 mM), and 0.25 µL *Taq* DNA polymerase (Qiagen) in a total volume of 50 µL. Amplification was checked by electrophoresis on 1.5% agarose gels stained with 0.2 mg^{-L} ethidium bromide.

2.10. Sequence Analysis

Amplified 16S rRNA gene fragments with primers 27F and 1492R were purified from impurities with a QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen). Sequencing reactions were performed with a BigDye Terminator cycle sequencing kit (Applied Biosystems) using the eubacterial primer 27F, and analyzed on an automatic sequencer (ABI PRISM 3100-Avant, Applied Biosystems). The resulting 16S rRNA gene sequences were compared with sequences deposited in GenBank by performing a blast n search. The phylogenetic relationship between Linuron-degrading species was studied with sequences obtained from GenBank. Sequences were aligned with the MEGA 4 software package [33] based on the ClustaLW algorithm [34] using default parameters, and Phylogenetic trees were generated with a neighbour-joining (NJ) algorithm. Confidence values for NJ trees were generated by bootstrapping, based on 1000 replicates.

2.11. Statistical Analysis

All the experiments were performed in triplicate and the average values with ±SD were reported in figures and tables. Separate statistical analysis (ANOVA) was done for each organism and different sets of experiments using Duncan's multiple range test (SPSS version 12.0) at 5% probability level ($P \leq 0.05$)

3. Results and Discussions

3.1. HPLC Profiles of Linuron Degradation

In the HPLC linuron was detected at the 5.4 min peak A (Figure 1a). Separate pure cultures of isolates LJk-5C, LWA-2C, LLa-1A, LoG-8A, LJk-5C and LWa-2A in MSM in which 50 mg/l of linuron was added were incubated at 28°C for 6 weeks. HPLC periodical monitoring of linuron degradation revealed new peaks B, C and E the peaks. The peaks continued to increase in area under the curve while, the area under linuron peak A continued to reduce an indication of linuron degradation (Figure 1b). At the 84th day of incubation (Figure 1c) several other metabolites F, G and H were detected at various retention times as the main compound appeared to diminish in concentration.
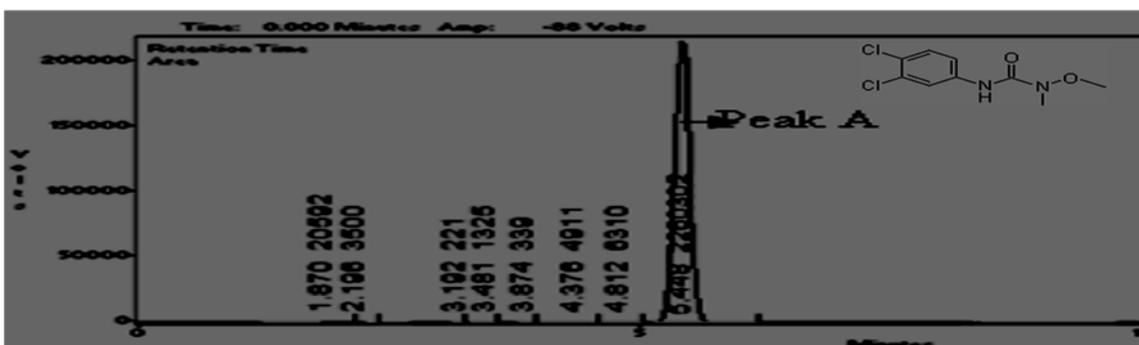
Figure 1a. HPLC profiles of linuron degradation on zero day. Peak A is linuron while the other peaks are mineral salts
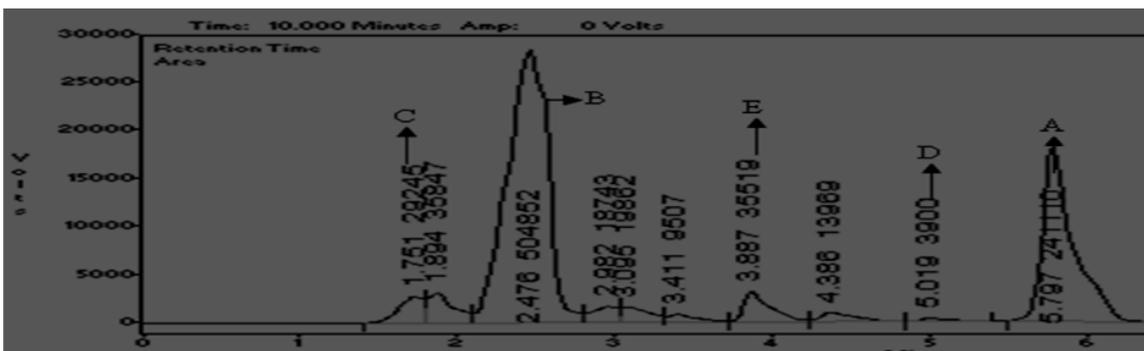

Figure 1b. HPLC profiles of linuron degradation on 42nd day. Peak A reduced while peaks B, C, D and E increased area
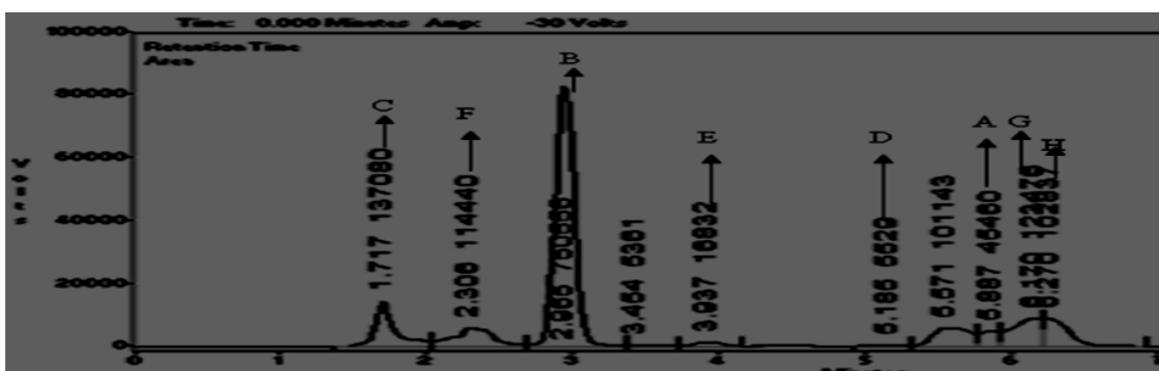

Figure 1c. HPLC profiles of linuron degradation on 84th day. Peak A was significantly reduced while peaks F, G and H were detected

3.2. Degradation Efficiency of Linuron in Pure Cultures

There was a long lag phase of 6 days in all bacterial strains. On the 7th day of incubation the level of quantifiable linuron degradation was observed by isolates Lsh-6B and LJk-5C which had a 13% degradation level in comparison to isolate LlA-1A had the lowest level of linuron removal of 3%. At the 14th day isolates LoG-8A, LWa-2A, Lsh-6B and LWa-2C had no significant difference ($P>0.05$) in the level of linuron degradation (Figure 2) while Isolate LJk-5C was observed to have the highest level of linuron degradation at 25.24%.
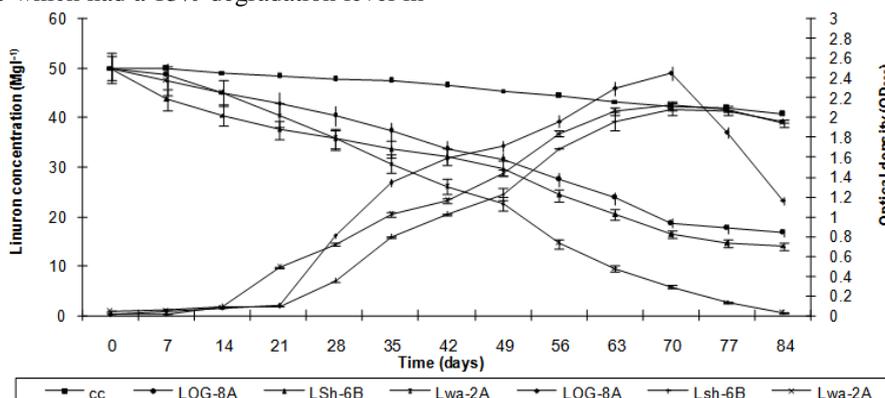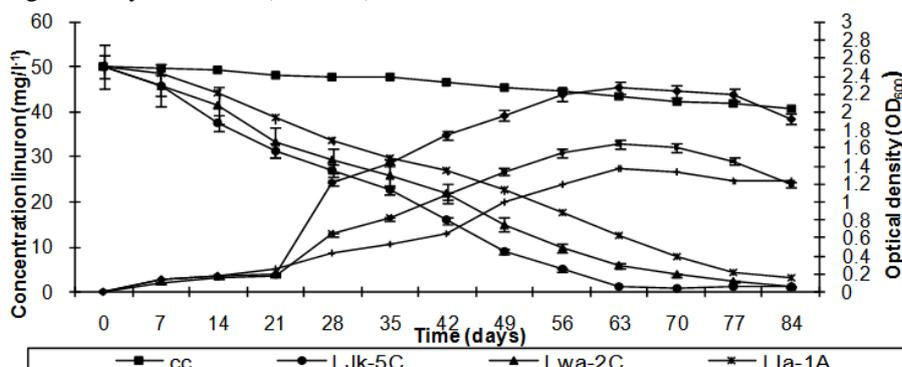

Figure 2. Degradation of linuron by isolate LOG-8A, Ljk-5B and Lwa-2A. The data points and error bars show the means and standard deviations

On the 21st day isolate LJk-5C recorded the highest level of linuron degradation of 37.74% while isolates Lla-1a and LoG-8A had no significant differences (P>0.05) in the level of linuron degradation and both isolates recorded the lowest level of linuron degradation of 9.58%. At the 28th day of isolates Lsh-6B and LWa-2A had the same level of linuron degradation of 30%, while LOG- 8A had the lowest degradation level of 20%. On the 35th day it was observed that the level of linuron degradation by the test isolates was significantly different (P< 0.05) with

isolate LJk-5C recording the highest level of degradation at 56% while isolate LoG-8A was observed to have the lowest degradation ability of linuron at 26% rate of removal. On the 42nd day isolates Lla-1A and LWa-2A had no significant difference (P>0.064) in the level of linuron degradation. Isolate LJk-5C was noted to be the fastest linuron degrading isolate with on 18% of the $50 mgL^{-1}$ of linuron remaining in the medium by the 49th day of incubation (Figure 3).

Figure 3. Degradation of Linuron by isolate Ljk-5C, Lwa-2C and Lla-1A. The data points and error bars show the means and standard deviations

On the 56th day concentration of Linuron in the control medium was 84% a reduction of only 16% while the concentration of Linuron in the slowest Linuron degrading isolate LoG-8A was 46%, an indication that the test organisms were the main route of Linuron loss from the culture medium. On the 84th LJk-5C had degraded Linuron to 0.32 mg/l, a reduction level of 99.3%. There were significant differences (P<0.05) in the level of Linuron degradation between each day over time among the test isolates. However, isolate LWa-2C showed no significant difference (P>0.076) in the level of Linuron degradation on the 77th and 84th day of incubation. The level of Linuron degradation by chemical processes observed in the control flask throughout the experimental period was only 18.6%.

3.3. GC-MS Identification of Linuron Degradation Intermediate Metabolites

Spectrums obtained from gas chromatography mass spetrometry indicated that microbial degradation of linuron resulted into five intermediate coumpounds (Table 1) in their degradation pathway. GC-MS analysis aided identification of linuron metabolites as: N-O dimethylhydroxylamine, 3-chloroanalline, 4-chloroanlline, 3, 4 dichloroanalline and N-methyl-N-methxy-carbamic acid. Strains degraded these metabolites selectively and none of the strains had the ability to degrade all the five metabolites simultaneously.

Table 1. Degradation Metabolites of Linuron

Linuron metabolites		
Compound Name	Retention time (min)	Molecular weight
N,O dimethylhydroxylamin	5.006	61
3-chloroanalline	10.008	127
4-chloroanlline	10.009	127
3,4 dichloroanalline	18.520	161
N-methyl-N-methxy-carbamic acid.	24.580	232

Table 2. Morphological and Biochemical Characterization of Linuron Degrading Isolates

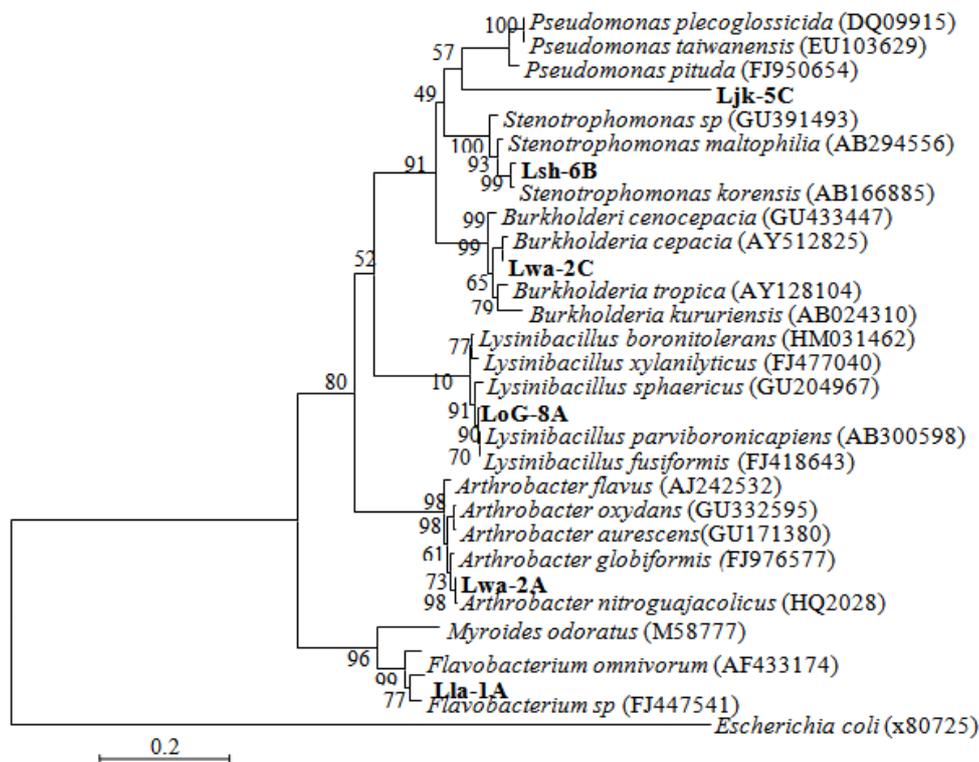|	Isolates					
Linuron Isolates	Lla-1A	Ljk-5C	Lwa-2A	LoG-8A	Lwa-2C	Lsh-6B
Cell shape	rod	rod	rod	rod	rod	Rod
Gram stain	+	-	+	+	+	-
motility	+	+	+	+	+	+
pigmentation	orange	Cream white	Cream white	yellow	brown	Pale yellow
Biochemical tests						
Oxidase	+	-	+	-	+	+
Catalase	+	-	-	+	-	+
urease	-	-	-	-	-	+
Nitrate reduction	-	+	-	-	+	+
TSI	K/K	A/A	K/K	K/K	K/K	A/A
MR	-	-	-	-	-	+
VP	+	-	+	-	+	-
Indole	-	-	+	-	-	-
Gelatinase	+	+	+	+	-	+

Legend: (+) Positive, (-): Negative, MR: Methyl Red, VP Voges-Proskeaur, TSI triple sugar iron, A/A; lactose and/or and glucose fermentation, KK No carbohydrate fermentation or hydrogen sulfide production.

3.4. Morphological and Biochemical Characterization of the Isolates

Linuron biodegradative isolates were characterized using morphological, cellular and biochemical characters as described by [15]. Gram test of the isolates was determined with 3% KOH (w/v) as described by [11]. The ability of the isolates to excrete extracellular enzymes was tested through hydrolysis of Tween 80 and gelatin. The ability of the isolates to excrete intracellular enzymes were determined through tests on sugars fermentation, hydrogen sulphide production, nitrate reduction, catalase reactions, urease, methyl red, voges-proskauer and triple sugar- iron test Table 2.

3.5. Phylogenetic Analysis of the Isolates

Figure 4. Phylogenetic tree showing position of isolate LoG-8A, Lwa-2A, Lsh-6B, LJk-5C, Lla-1A and Lwa-2C. The scale bar indicates approximately 2% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling. The 16S rRNA gene sequence of *Escherichia coli X80725* was used as an out group

Phylogenetic analysis showed that the isolates clustered into genera namely; *Pseudomonas sp, Stenotrophomonas sp, lysinibacillus sp, Arthrobacter sp Myroides sp* and *Burkholderia sp* for linuron degraders. Phylogenetic clustering pattern of isolate LoG-8A on the phylogenetic tree (Figure 4) indicated that the isolate clustered with *Lysinibacillus sp.* blasting further revealed that LoG-8A had 98% similarity to *lysiniBacillus sphaericus.* Isolate Lwa-2A clustered closely to *arthrobacter nitroguajacolicus* with a sequence similarity of 99%. Clustering pattern of isolates Lwa-2C, Lsh-6B, Ljk-5C and Lla-1A that they clustered with the genus *Burkholderia, Stenotrophomonas, Pseudomonas* and *Myroides sp* respectively supported by high bootstrap values of between 50 - 100%.

4. Discussion

The identity of the six isolates through a polyphasic approach revealed that the isolates were members of the genera *lysinibacillus, Burkholderia, pseudomonas, Arthrobacter Flavobacterium and Stenotrophomonas.* Phylogenetic analysis allowed identifying strain Ljk-5C as a *Pseudomonas* sp. The genus Pseudomonades have in earlier studies been reported to degrade aromatic hydrocarbons and pesticide [26]. In the study HPLC analysis showed that isolate Ljk-5C had the highest removal rate of linuron at 99.3% over an incubation period of 84 days. GC-MS analysis showed that the isolate also had the ability to degrade one of the intermediate metabolite 4CA with a removal level of 83.8% within 38 days of culture compared to other aniline however, metabolism of this intermediate was too slow after the 38th day of the experiment this could have been as a result of inhibition by 4-chlorocatechol a metabolite of the complete degradation of 4CA has previously reported by [32]. Isolate Lsh-6B was identified as a *Stenotrophomonas sp,* the genus Phylogenetic position of the isolate showed that it clustered more closely to *Stenotrophomonas maltophila.* Strain Lsh-6B had linuron degradation rate of 71.7% over the 84 days experimental period with accumulation of 3, 4 DCA in the medium. GCMS profiles revealed absence of 4CA metabolite in the cultural vessel of isolate at the end of the incubation period. These results suggests that the isolates had the ability to degrade 4-chlorocatechol an intermediate compound which had earlier been reported to be rate-limiting step in the metabolism of 4CA by *pseudomonas sp* following the ortho-cleavage pathway [6].

Isolate Lwa-2C was indentified to *Burkholderia sp* with 99% sequence similarity to *Burkholderia cenocepacia.* The isolate had 95.5% removal percentage of linuron by end of 84 day incubation period however, GC-MS

analysis revealed that the strain was not able to further breakdown other intermediate metabolite of linuron degradation. In earlier reports many members of genus *Burkholderia* have been discovered as pesticide degrader, species like *Burkholderia kururiensis, Burkholderia sacchari, Burkholderia terrae* and *Burkholderia cepacia* [2-14].

Phylogenetic analysis of isolate LLa-1A showed that the isolate belonged to genera *Myroides.* The sequences displayed the highest identity (97%) with the 16S rRNA gene of a *Flavobacterium sp.* The isolate had a 93.8% removal capacity of linuron at the 84th day of incubation. On anilines degradation the isolates had the ability to metabolize 3CA and 4CA at a removal rate of 18% and 11% respectively however, 3, 4 DCA was observed to accumulate in the cultural medium. The concerted action of *Myroides sp* has already been observed for degradation of atrazine by [30] who reported that *Myroides sp* had the capacity to degrade atrazine herbicide with a removal percentage of 90% within 3 days. A study by [10] revealed that atrazine-degrading genes were highly conserved, widespread and frequently associated with insertion sequences (IS) located on plasmids consequently, and the combination of IS-mediated rearrangement and plasmid transfer had been suggested to contribute to the assembly and dissemination of the atrazine-degrading capabilities in the environment. From these earlier findings it may be possible for these genes to have contributed to strain LLa-1A capability to degrade the study compound linuron.

Isolate LWa-2C, 16S rRNA gene sequencing and blast analysis indicated that Lwa-2A was 99% homologous to 16S rRNA gene of *Arthrobacter* sp. Lwa-2A was closest to *Arthrobacter nitroguajacolicus*. The isolate degraded 98.9% of 50mg/l of linuron over a period of 84 days. In other related studies *Arthrobacter sp* have been reported to metabolize methoxy-methyl-substituted phenylureas such as linuron, monolinuron and metobromuron but not diuron and monuron [8-38]. Phylogenetic analysis clustered isolate LOG-8A with *Lysinibacillus sp*. Isolate LOG-8A showed 62.7% reduction in concentration of linuron by the 70th day of incubation which was followed by a sharp decline in cell mass of the isolate an indicator of cell death. The observation agrees with the work of [9] who isolated *Bacillus sphaericus* ATCC 12123 that could use the alkyl chain of linuron as the sole N and C source. However, he also observed that these pure strain could only partially degrade linuron, since accumulation of (3, 4-DCA) become toxic to the strain.

5. Conclusions

A better understanding of the mechanism of biodegradation has a high ecological significance that depends on the indigenous microorganisms to transform or mineralize the organic contaminants. Microbial degradation process aids the elimination of linuron from the environment in a cost effective way. This is possible because microorganisms have enzyme systems to degrade and utilize herbicides as a source of carbon and energy. The knowledge of microbial degradation of linuron may serve as a basis for the use of bioremediation systems for the removal of other herbicides in near future.

References

[1] Amine-Khodja., A. Boulkamh, A. and Boule., P. "Photochemical behavior of phenylurea herbicides." *Photochemical and photobiology science*. 3: 145-156. 2004.

[2] Anthony, R., Smith, W., and Carol, A, "Induction of enzymes of 2, 4 dichlorophenoxyacetate degradation in *Burkholderia cepacia 2a* and toxicity of metabolic intermediates", Biodegradation., 19, (5). 669-681. Sep. 2008.

[3] Alexander, K. Steve., K., and Strete. D, *Microbiology: a photographic atlas for the laboratory*. Benjamin Cummings publishers, California, 2001.

[4] Benitez, F.J., Acero, J.L., Real, F.J., and Garcia, C, "Removal of phenyl-urea herbicides in ultrapure water by ultrafiltration and nanofiltration processes". *Water Research*, 43 (2) 267-276. Feb 2009.

[5] Birech, R., Bernhard, F., and Joseph, M.. "Towards reducing synthetic pesticide imports in favour of locally available botanicals in Kenya" in the Conference *on International Agricultural Research for Develop,* Bonn Germany 8-12.

[6] Boon, N., Goris, J., De Vos, P., Verstraete, W., and Top, E. "Genetic diversity among 3-chloroaniline-and aniline-degrading strains of the *Comamonadaceae"*. *Appllied and Environmental Microbiology*. 67 (3) 1107-1115. Mar 2001.

[7] Caux, P.Y., Kent, R. A., Fan, G. T., and Grande, C. "Canadian water quality guidelines for Linuron." Environ mental Toxicololgy and Water Quality 13 (1). 1-41. Dec 1998.

[8] Cullington, J., and Walker A. "A rapid biodegradation of diuron and other phenylurea herbicides by a soil bacterium". *Soil Biolology and Biochemistry.* 31 (5). 677-686. May 1999.

[9] Dejonghe, W., Goris J., Dierickx, A., De Dobbeleer, V., Crul, V., De Vos, P., Verstraete, W., and Top, E. "Diversity of 3-chloroaniline and 3,4-dichloroaniline degrading bacteria isolated from three different soils and involvement of their plasmids in chloroaniline degradation." *FEMS microbiology and ecology*. 42 (2). 315-325. Jan 2006.

[10] Devers, M. Azhari, N.E. Udikovic Kolic, N. and Martin Laurent, F. Detection and organization of atrazine degrading genetic potential of seventeen bacterial isolates belonging to divergent taxa indicate a recent common origin of their catabolic functions, *FEMS Microbiology Letter*s 273 (1) 78-86. June 2007.

[11] Gregersen T, "Rapid method for distinction of Gram-negative from Gram-positive Bacteria". *European Journal of applied Microbial Biotechnology* 5 (2) 123-127. 1978

[12] Gooddy, D.C., Mathias S.A., Harrison I., Lapworth. D.J. and Kim A.W, "The significance of colloids in the transport of pesticides through Chalk." *Science Total Environment.* 385 (1-3) 262-271. Oct 2007.

[13] Guzzella, L., Capri, L., DiCorcia, A., Barra Caracciolo, A. and Guiliano, G. "Fate of Diuron and Linuron in a Field Lysimeter Experiment." *Journal of Environmental Quality* 35: 312-323. 2006.

[14] Hiroaki, I., Kazuya, A. and Yoshie, H. "Isolation and characterization of a new 2, 4-dinitrophenol-degrading bacterium *Burkholderia* sp. strainKU-46 and its degradation pathway." *FEMS Microbiology Letters,* 274 (1). 112-117. Sep. 2007.

[15] Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. and Williams, S. T. Bergey's Manual of Determinative Bacteriology, 9th Ed. Williams and Wilkins publishers, London, 1994.

[16] Johnson, A. Besien, T. Bhardwaj, C. Dixon A, Gooddy, D. Haria, A. and White, C. "Penetration of herbicides to groundwater in an unconfined chalk aquifer following normal soil applications." *Contaminant hydrology*. 53: 101-117. 2001.

[17] Lambright, C., Ostby J., Bobsiene K., Wilson V., Hotchkiss K., Mann C. and Gray E, " Cellular and Molecular Mechanisms of action of Linuron an Antiandrogenic Herbicide that Produces Malformations in Male Rats". *Toxicological Science,* 56 (2). 389-399. Aug 2000.

[18] Lamoree, M., Swart, C., van der Horst, A. and van Hattum B. "Determination of diuron and the antifouling paint biocide

Irgarol1051 in Dutch marinas and coastal waters." *Journal of Chromatography A,* 970: 183-190, 2002.

[19] Macharia, I., Mithofer, D.and Waibel, H. "Potential environmental impacts of pesticides use in the vegetable sub-sector in kenya." *African Journal of Horticultural Science* 2, 138-151. 2009.

[20] Marmur, J. "A procedure for the isolation of deoxyribonucleic acid from micro-organisms." *Journal of Molecular Biolology* 3: 208-218. Jan 1961.

[21] Mehrdad, E., Wabule, M.N., Ngaruiya, P.N., Kimmins, F.K. and Silverside, P.J. "Problems facing the flower industry and registration for Biocontrol Agents in Kenya," in *Proceedings of the PCPB/KARI/DFID CPP Workshop, Nakuru, Kenya,* 14-16 May 2003.

[22] Muhammad, S., Asif, T., Muhammad, A., Khuram, M., Naeem, S., Muhammad, I. and Imran, Q. "Effect of weed-crop competition on growth and yield of garden cress *Lepidium sativum* L". *Journal of Medicinal Plants Research*. 5 (26), 6169-6172. Nov 2011.

[23] Philip, B., Pieter-Jan, D., Rene De, M., and Dirk, S. "Characterization of novel linuron-mineralizing bacterial consortia enriched from long-term Linuron-treated agricultural soils." *FEMS microbial and ecology* 62: 374-385. Nov 2007.

[24] Polz, M. and Cavanaugh, C. "Bias in template-to-product ratios in multitemplate PCR." *Applied Environmental Microbiology* 64 (10) 3724-3730. Oct 1998.

[25] PCPB (2005). Pest Control Products Board, July, 2004-June, 2005 Annual Report.

[26] Ramanathan, M. and Lalithakumari, D. "Complete mineralization of methylparathion by Pseudomonas sp. A3". *Applied biochemistry and biotechnology*. 80 (1) 1-12. April 1999.

[27] Ritter, R. Scheringer, M. MacLeod, M. Moeckel, C. Jones, K. and Hungerbühler K. "Intrinsic human elimination half lives of polychlorinated biphenyls derived from the temporal evolution of cross-sectional biomonitoring data from the UK population." Environmental Health Perspect 119: 225-231.2011

[28] Richardson, M. "Pesticides—friend or foe?" *Water Science and Technology* 37 (8) 19-25. August 1998.

[29] Scassellati-Sforzolini, G. Pasquini, R. Moretti, M. Villarini, M. Fatigoni, C. Dolara, P. Monarca, S. Caderni, G. Kuchenmeister, F. Schmezer, P. and B. L. Pool-Zobel. "*In vivo* studies on

genotoxicity of pure and commercial linuron. Mutat. Res. Genet. Toxicol." *Environ mental Mutagen.* 390 (3) 207-22. 1997.

[30] Smith, D. Alvey, S. and Crowley, D. "Cooperative catabolic pathways within an atrazine degrading enrichment culture isolated from soil.' *FEMS Microbiology and Ecology.* 53 (2): 265-273. Jul 2005.

[31] Sorensen, S.R., Bending, G.D., Jacobsen, C.S., Walker, A. and Aamand, J. "Microbial degradation of isoproturon and related phenylurea herbicides in and below agricultural fields." *FEMS Microbiology of Ecology,* 45 (1) 1-11. July 2003.

[32] Surovtseva, E. G. Sukhikh, A. P. and Ivoilov, V. S. "Isozymes of the pathway for aniline and 4-chloroaniline preparatory metabolism in *Alcaligenes sp. Mikrobiologiy,* 61: 99-106. 1993

[33] Tamura, K. Dudley, J. Nei, M. and Kumar, S. "MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0." *Molecular Biology and Evolution* 24 (8): 1596-1599. August 2007

[34] Thompson, J. Higgins, D. and Gibson, T. "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." *Oxford journal of nucleic acid research.* 22 (22) 4673-4680. Sep 1994.

[35] Tixier., C. Sancelme., M. Bonnemoy., F. Cuer., A. and Veschambre., H. " Degradation products of a phenylurea herbicide, diuron: synthesis, ecotoxicity, and biotransformation*." Environmental Toxicology & Chemistry*, **20:** 1381-1389, 2001.

[36] U.S. Environmental Protection Agency (U.S. EPA) (1995) R.E.D. Facts Linuron (EPA-738-F-95-003). pp. 1-11 (http://www.epa.gov).

[37] Uyttebroek, M. Breugelmans, P. Janssen, M. Wattiau, P. Joffe, B. Karlson, U. Ortega-Calvo, JJ. Bastiaens, L. Ryngaert, A. Hausner, M. Springael, D. "Distribution of the Mycobacterium community and polycylic aromatic hydrocarbons (PAHs) among different size fractions of a long-term PAH-contaminated soil." Environmental Microbiology 8 (5) 836-847. May 2006.

[38] Ware, G.W. and Whitacre, D.M. *The Pesticide Book*, MeisterPro Information Resources*,* Willoughby, Ohio. pp. 496.

[39] Widehem P, Ait-Aissa S, Tixier C, Sancelme M, Veschambre H and Truffaut, N. " Isolation, characterization and diuron transformation capacities of a bacterial strain *Arthrobacter sp.* N2." *Chemoshere*, 46 (4) 527-534. Jan 2002.