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Isolation and Identification of Hexazinone-Degrading Bacterium from Sugarcane-Cultivated Soil in Kenya

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Abstract The s-triazine herbicide hexazinone [3-cyclohexyl-6-dimethylamino-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione], is widely used in agriculture for weed control. Laboratory biodegradation experiments for hexazinone in liquid cultures were carried out using sugarcane-cultivated soils in Kenya. Liquid culture experiments with hexazinone as the only carbon source led to the isolation of a bacterial strain capable of its degradation. Through morphological, biochemical and molecular characterization by 16S rRNA, the isolate was identified as *Enterobacter cloacae*. The isolate degraded hexazinone up to 27.3 % of the initially applied concentration of 40 μg mL^{-1} after 37 days of incubation in a liquid culture medium. The study reports the degradation of hexazinone and characterization of the isolated bacterial strain.

Keywords Hexazinone · Biodegradation · Herbicide · Liquid cultures · *Enterobacter cloacae*

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Hexazinone is a herbicide that is effective for general weed control in agricultural and non-agricultural areas (Zhu and Li 2002). Since hexazinone is tolerated by conifers, it is a very effective herbicide for reducing competition from broad leaf trees and bushes, as well as annual and perennial weeds (Ghassemi et al. 1981). Hexazinone is highly soluble in water (33 g L^{-1}), and has a low average organic carbon adsorption coefficient ($K_{oc} = 610$) and a low octanol/water coefficient ($K_{ow} = 15.0$). Therefore, it is mobile in the environment with a great potential for leaching and partitions into water more than to soil, or biota (Wang et al. 2006; Ganapathy 1996). With the moderate to long half-life and high mobility, hexazinone can potentially move off-site with water in run-off and in base flow. The average half-life for hexazinone has been reported as 90 days (Tu et al. 2001). USDA-ARS, (2002) lists a value of 79 days, whereas DuPont Fact sheet (1999) gives a half-life of 175 days. In Kenya, studies have shown the presence of pesticide residues in sugarcane cultivated soils and water from the drainage basins (including Lake Victoria) in sugarcane farming regions, hence the growing concerns regarding the potential effects of pesticides on non-target organisms (Getenga et al. 2004). Hexazinone presents toxicological hazards and, in particular, is toxic to aquatic organisms and may cause long term adverse effects in the aquatic environment (Pang et al. 2005).

Sugarcane farming is a major economic activity in Kenya, with the industry supporting over 5 million people (Kenya's Sugar Industry 2005). In sugarcane farming, weeds have been estimated to cause 30 %–70 % reduction in cane yield depending upon the severity of infestation, thereby necessitating the heavy use of herbicides in their control (KESREF 2009). In Kenya, hexazinone is used under the trade name Velpar 75 DF^{TM} and applied at a rate of 0.5 or 1 kg ha^{-1} (or more in fields where the weeds have

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developed resistance) for the control of pre- and post-emergence broad-leafed and grassy weeds in sugar cane fields.

Biodegradation is one of the most important environmental fates of herbicides that aids in their removal from the soils. Hexazinone is primarily degraded through microbial metabolism in soils and is not significantly affected by photo or chemical degradation (Wang et al. 2006). Some studies have reported the degradation behaviour of hexazinone in soils (Wang et al. 2006; Rhodes 1980; Mostafa and Heilling 2003). Its degradation in soil is primarily by demethylation and hydroxylation of the cyclohexyl ring (Rhodes 1980). Among the triazines, atrazine is the most widely studied herbicide. Studies that report on biodegradation of hexazinone are few, yet its use has increased due to the restrictions imposed on atrazine. In Kenya, no hexazinone-degrading microbial communities or single strains have been isolated or characterized. The objective of this study was therefore to investigate the hexazinone degradation capacity of a soil from a sugarcane field with application history, to enrich the degrading microbial community from this soil and to isolate and identify the key degraders.

Materials and Methods

Soil samples (0–10 cm layers) were collected from sugarcane cultivated field in Nzoia sugar company nuclear estate (located at 34°34′10″–34°51′32″E and 0°23′07″–0°37′45″N) in Western Kenya. The field had a history of hexazinone application for over 5 years. The field had clay loamy soils composed of 40 % sand, 33 % clay and 27 % silt content. The soil nitrogen content (% N) was 0.15, 1.5 % organic C, 2.89 mg kg^{-1} P, 19.17 mg kg^{-1} S, 138 mmol kg^{-1} CEC and a pH of 4.6 ($CaCl_2$). Before the experiments were started, the soil samples were sieved (<2 mm) and kept at room temperature for 5 days after moistening.

The residual hexazinone in soils was determined prior to laboratory degradation experiments. A 10 g subsample of the homogenous air-dried soil sample was weighed in triplicate and extracted continuously by soxhlet method with 25 mL methanol for 8 h (Bicalho et al. 2010; USEPA 1996). The whole extracts of 25 mL were dried by use of anhydrous sodium sulfate, filtered through 0.45 μm polytetrafluoroethylene (PTFE) filters and cleaned-up with Isolate Triazine columns (500 mg, Separtis, Grenzach-Wyhlen, Germany). After extraction, the SPE columns were dried under a gentle nitrogen-stream and eluted with 10 mL methanol. The eluate was concentrated to a volume of 1 mL with a rotary evaporator and further concentrated to a volume of 0.2 mL under a gentle nitrogen-stream. Extracts were analysed by HPLC (LC-10AT VP Shimadzu, Japan). The HPLC was equipped with SPD-10A VP Shimadzu UV–VIS detector, 125 × 4 mm Crom Saphir 110 C_{18} 5 μm column. The measurements were conducted at the following conditions; Mobile phase (isocratic) acetonitrile: water (60:40) mixture at a flow rate of 1 mL min^{-1}, injection volume of 20 μL and the detector was set at 247 nm. Pestanal grade hexazinone reference standard was used.

Mineral salt medium (MSM) [20 mM K_2HPO_4; 10 mM KH_2PO_4; 1.1 mM $MgSO_4{\cdot}7H_2O$; 3.12×10^{-2} mM $Fe_2(SO_4)_3{\cdot}9H_2O$; 2.32×10^{-1} mM $CaCl_2{\cdot}2H_2O$], was prepared and sterilized. Iron sulfate was filter-sterilized and added to the medium after autoclaving. Aliquots of 49 mL of the sterile MSM were amended with 40 μg mL^{-1} hexazinone. An aliquot of 1 g of soil was suspended in 9 mL of 0.85 % NaCl in de-ionized distilled sterile water, vortexed for 2 min and the mixture diluted to 10^{-3} in ten-fold dilution steps. Dilutions of the soil samples (aliquot of 1 mL) were used for inoculating the 49 mL MSM with hexazinone as the sole carbon and nitrogen source in 100 mL-Erlenmeyer flask. The liquid cultures were incubated (in four replicates) at 27 ± 1°C in the dark with shaking at 120 rpm. An aliquot of 3 mL was withdrawn periodically at two to three day-interval to measure the optical density (OD_{600}). The same aliquot was used for HPLC analysis of residual hexazinone and metabolites after filtration through 0.2 μm filters. A plot of optical density and degradation expressed as residual hexazinone remaining against time was obtained.

Liquid cultures which showed enhanced degradation of hexazinone were subjected to further liquid culture enrichment experiments. Aliquots of 49 mL of MSM (freshly prepared) were transferred to sterile100-mL flasks in a sterile hood and inoculated with 1 mL aliquots of the selected previous liquid cultures with enhanced hexazinone degradation capacity. A series of new liquid cultures were prepared from time to time to enrich the liquid cultures with hexazinone degraders by using inoculants from the previous liquid culture. From the 5th enrichment of the mixed liquid culture, an aliquot of 100 μL was taken and diluted to 1 mL with a fresh MSM. Serial dilutions were made up to the 10^{-3} dilution in sterile eppendorf tubes. MSM with hexazinone as the sole carbon and nitrogen source was prepared and solidified with 15 g L^{-1} agar. Aliquots of the medium were poured on the plates and left to cool. From the 1st and 3rd dilutions, 100 μL aliquots were spread on the agar plates in duplicates and the plates incubated at 30°C. The individual colonies were separately streaked on another set of agar plates and incubated at 30°C. A total of seven bacterial isolates were obtained from the MSM on the agar plate amended with 40 μg mL^{-1} of hexazinone.

The seven colonies were individually suspended as pellets in liquid culture medium amended with 40 μg mL^{-1} hexazinone and incubated (in four replicates) for 37 days at

27 ± 1°C in the dark with shaking at 120 rpm. The MSM medium without inoculums but with hexazinone and an inoculated MSM medium without hexazinone were used as controls. Turbidity and concentration of hexazinone were periodically measured after every 2–3 days. The aliquot of 3 mL withdrawn from the liquid cultures was centrifuged at 5,000 rpm for 10 min at 25°C, filtered and then used for HPLC analysis. The degradation rate was determined by regression using first-order rate equation $C = C_0^{-kt}$; where C is concentration of hexazinone remaining in liquid medium (μg mL^{-1}), k is degradation rate (day^{-1}), C_0 is the initial concentration of hexazinone in liquid medium (μg mL^{-1}) and t is time in days (Tortella et al. 2010).

Colony morphology was determined using a dissecting microscope. Biochemical tests included tests on ability to utilize the following compounds as the sole source of carbon: glucose, arabinose, sucrose, lactose, D-mannitol, D-galactose, D-mannose, maltose and fructose. Other biochemical tests included nitrate reduction, citrate utilization, catalase and oxidase tests, H_2S production, indole test, methyl red (MR) test, motility test, Voges-Proskauer (VP) test, casein and starch hydrolysis, and hydrolysis of urea (Cappuccino and Sherman 2002). The identity of the isolates was further confirmed through molecular characterization. Total bacterial DNA was extracted according to procedures described by Schmidt et al. (1991), purified and used as a template for amplification of 16S rRNA gene. Total DNA samples extracted from the isolates were detected using gel electrophoresis. The 16S rRNA gene sequences were PCR-amplified using bacterial primer pair 27F forward 5′-GAG TTT G(AC)T CCT GGC TCA G-3′) and 1492R reverse, 5′-TAC GG(CT) TAC CTT GTT ACG ACT T-3′ (Eurofins MWG GmbH). Amplification was carried out in a 30 μL mixture containing 3 μL of 10× PCR buffer, 4 μL of 2.5 mM dNTPs, 2.5 μL of 27F forward primer (5 pmol), 2.5 μL of 1492R reverse primer (5 p mol), 0.4 μL of 5 U/μL *Taq* polymerase, 1.5 μL template DNA and 16.1 μL of PCR grade water. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, primer annealing at 55°C for 50 s, chain extension at 72°C for 90 s, and a final extension at 72°C for 8 min. Amplification products (5 μL) of each DNA sample was loaded on an ethidium bromide containing agarose gel (1 %) in 1× TAE buffer and run at 70 V for 1.5 h. The PCR amplicons (≈15 kb) from the isolates were excised from the gel and purified using quickClean 5 M gel Extraction kit (GenScript Corporation, 120 Centennial Ave, Piscataway, NJ 08854) according to the manufacturer's instructions. The sequencing of the 16S rRNA genes was done at BECA (Biosciences Eastern and Central Africa) in ILRI (International Livestock Research Institute), Nairobi

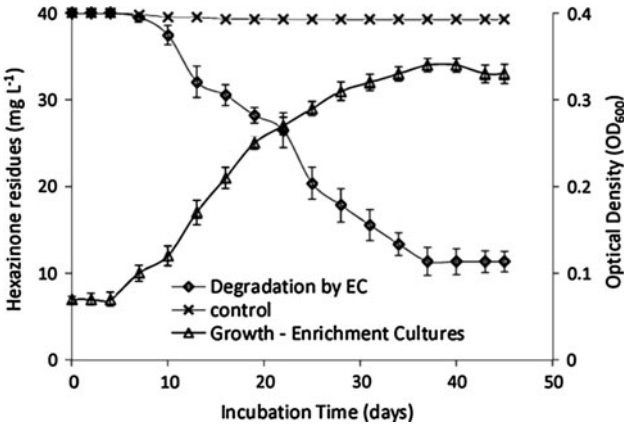

Fig. 1 Bacterial growth and hexazinone degradation curves in liquid enrichment cultures (EC) with initial hexazinone concentration of 40 μg mL^{-1}. Control contained uninoculated media. *Bars* indicate standard deviation of four replicates

using 27F forward 1492R reverse primers as specified in PCR amplification. Sequencing was conducted with Big-Dye™ Terminator technology. The sequencing products were run on ABI 3130 Genetic Analyzers. The forward and backward 16S rRNA gene sequences of the bacteria isolates were viewed and edited using Chromas software package (www.technelysium.com.au). They were then aligned using Bioedit sequence alignment editor software package (Hall 1999) to provide full sequences of about 1,500 nucleotide bases. The sequences were compared with sequences in the public databases with the BLAST search program on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16S rRNA gene sequences. The ARB database software package (Ludwig and Strunt 1996) was used to align and identify the closely related bacterial 16S rRNA gene sequences. The 16S rRNA gene sequences of the isolates and those of the closely related bacteria were then aligned and processed to produce a phylogenetic tree using MEGA software package (www.megasoftware.net).

The statistical packages, GenStat Discovery edition 3 and Microsoft Excel 2007 were used for data analysis.

Results and Discussion

The soil had residual hexazinone of 0.75 ± 0.20 μg g^{-1}. The adopted method of extraction provided recoveries of 94 % (±3.1). The limit of detection (LoD) for hexazinone was 0.018 μg g^{-1} soils (dry weight) for the adopted method of residual herbicide analysis. The residual hexazinone obtained may have been due to the extensive use of this herbicide in sugarcane production coupled with an elevated application rate of more than 1 kg ha^{-1}.

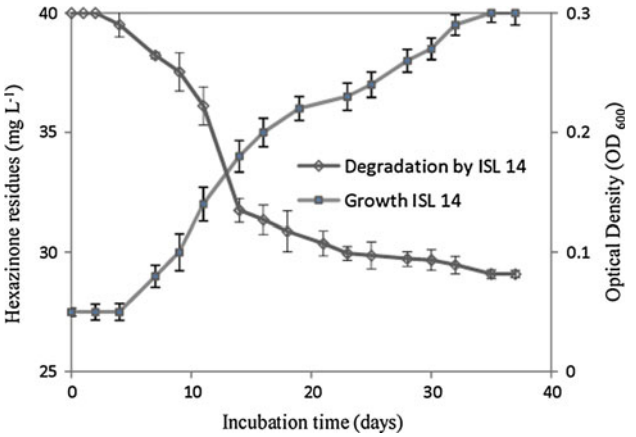

Fig. 2 Bacterial growth and hexazinone degradation curves in liquid culture by isolate ISL 14 with initial hexazinone concentration of 40 μg mL^{-1}. *Bars* indicate standard deviation of four replicates

The liquid enrichment culture degraded hexazinone up to 71.7 % of the applied amount. Growth in the cultures increased with hexazinone degradation (Fig. 1) as indicated by optical density measurements. The enrichment culture showed a lag phase of 7 days and reached its highest at an OD_{600} of 0.33 on the 45th day of incubation.

Liquid culture enrichment experiment for hexazinone yielded seven bacterial isolates but only one isolate, ISL 14, was capable of degrading hexazinone. This isolate degraded hexazinone up to 27.3 % of the applied amount after 37 days incubation (Fig. 2).

Degradation of hexazinone by isolate ISL I4 resulted in the formation of two detectable metabolites identified as [3-(4-hydroxycyclohexyl)-6-(dimethylamino)-l-methyl-l,3,5-triazine-2,4(1H,3H)-dione (A) and [3-(4-hydroxycyclohexyl)-6-(methylamino)-l-methyl-l,3,5-triazine-2,4(1H,3H)-dione (B). Results showed formation of 6.3 % A and 10.5 % B (% of applied amount of hexazinone) after 14 days of incubation. These amounts reduced to 1.5 % and 3.2 % for metabolites A and B, respectively after 37 days of incubation. The degradation pattern for hexazinone fits a first order degradation kinetics ($R^2 = 0.9309$) with a degradation rate of 0.011 μg mL^{-1} hexazinone per day in liquid cultures. However, the degradation rate of the pure isolate was low compared to that of the enrichment cultures (0.034 μg mL^{-1} per day). This may suggest that complete degradation is by a consortium of microorganisms thus leading to loss of synergism after the isolation of a single strain. This consortium of microorganisms represents apparent "non-culturable" components of the degrading microbial community. The formation of the metabolites was compared with the amount of degraded hexazinone. There was a significant positive correlation ($p < 0.0001$) between metabolite B and hexazinone degradation. The formation of this metabolite was as result of microbial demethylation and hydroxylation of the cyclohexyl ring of hexazinone (Ganapathy 1996).

Morphological determinations showed that isolate ISL I4 was rod-shaped and gram-negative. The isolate was motile, oxidase-negative and catalase-positive. It gave positive results in MR test, citrate utilization and in nitrate reduction. It gave negative results in casein hydrolysis, starch hydrolysis, urease, VP, indole tests and in H_2S production. The isolate was able to utilize all the tested sugars; lactose, sucrose, glucose, arabinose, D-mannitol, D-galactose, maltose, and fructose but not mannose. These tests results were similar to that of an *Enterobacter cloacae* strain reported by Binks et al. (1996). Phylogenetic analysis of 16S rRNA gene sequences of isolate ISL14 showed that the isolate clustered with the genus *Enterobacter*. This clustering was supported by high bootstrap values of between 66 % and 100 % (Fig. 3). The isolate had 16S rRNA gene sequence similarity of 96 % to *E. cloacae* FJ527682.

The degradation of hexazinone has not been as extensively studied as atrazine, a commonly used s-triazine herbicide. However, some bacterial strains have been isolated that are capable of biodegrading hexazinone. Studies by Wang et al. (2006) identified *Pseudomonas* sp. and *E. cloacae* strains as hexazinone degrading bacterial strains from subtropical soil. The two isolates degraded hexazinone rapidly with half-lives of 3.08 and 2.96 days, respectively, compared to a half-life of 63 days by the isolated *E. cloacae* in the current study. However, it was also observed that the mixed bacterial culture degraded hexazinone at a higher rate compared to individual isolates. This observation concurs with the results of the current study whereby the enrichment culture showed a higher degradation rate compared to the isolated bacterium. Mostafa and Heilling (2003) identified *Microbacterium foliorum, Paenibacillus illinoisensis* and *Rhodococcus equi* as hexazinone degraders from tropical soils. This study reports for the first time the degradation of hexazinone by *E. cloacae* in tropical soils.

Some previous studies have reported the isolation of *E. cloacae* and other *Enterobacter* strains from different sources that were capable of degrading diverse pollutants (Singh et al. 2004; Kafilzadeh et al. 2012). Shapir et al. (2006) showed that allophanate hydrolase from *E. cloacae* strain 99 metabolized cyanuric acid, the intermediate in atrazine metabolism, to allophanate and then finally to CO_2 and NH_3. A study by Ngigi et al. (2012) had identified *E. cloacae* as an atrazine degrader. Such studies strongly support the developed capability of various *Enterobacter* strains in degradation of xenobiotics as also indicated by the findings of this study. The isolation and identification of *E. cloacae* as a hexazinone degrader adds information on fate of the herbicide in tropical soils. Further investigations on the identified degrader may be carried out for enhanced degradation and decontamination of soils.

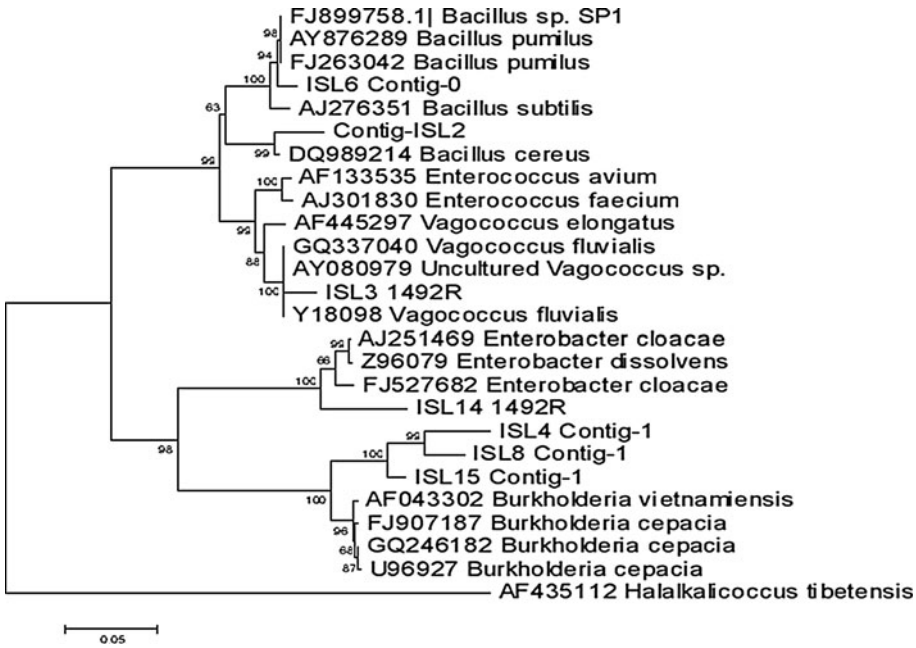

Fig. 3 Phylogenetic tree for bacterial isolates. The *scale bar* indicates approximately 5 % sequence difference. *Numbers at nodes* indicate bootstrap values of each node out of 100 bootstrap resampling. The out group 16S rRNA gene sequencing of *Halalkalicoccus tibetensis* AF435112 was used as an out group

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