U. of K. J. Agric. Sci. 21(2), 233-252, 2013

Biodegradation of Pendimethalin by Three strains of Bacteria Isolated from Pesticide-Polluted Soils*

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Abstract: Biodegradation of pendimethalin by three strains of bacteria viz; *Pseudomonas aeruginosa, Bacillus mycoides* and *Bacillus cereus* isolated from pesticides polluted soil was studied in mineral salt medium. Pendimethalin was incubated with each of the three strains of bacteria and samples were drawn at 0, 3, 7, 15 and 30 days for gas chromatographic analysis. The loss in the initial concentration (400 ppm) over time was monitored and used to compute the half lives following a biphasic model. GC-MS was used to identify the major metabolites as well as to re-confirm the identity of the starting material (pendimethalin). The results showed that the remaining amounts of pendimethalin recovered from media inoculated with *Pseudomonas aeruginosa* for 3, 7, 15 and 30 days were; 75.5%, 69.25%, 29.75 % and 19.25% respectively, While the amounts recovered from media inoculated with *Bacillus mycoides* were; 48.75%, 46.25%, 39.25 % and 28.25% following the same order. On the other hand, the respective amounts recovered from media inoculated with *Bacillus cereus* were; 45 %, 32.5 %, 30.5 % and 19.75% at 3, 7, 15 and 30 days, respectively. Despite the significant drop in the starting material, no metabolites were detected *in Bacillus* cultures while only *N*-(1-ethylpropyl)-3-methyl-2, 6-diaminobenzine was detected in *P. aeruginosa* culture indicating the capability of these microorganisms of complete mineralization of pendimethalin. Pendimethalin biodegradation by the three types of bacteria followed a biphasic model with faster rate of

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* Part of M, Sc. thesis by the first author submitted to the University of Khartoum, Shambat, Sudan

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disappearance in the first phase and slower rate in the second. The half lives for the first phase ranged from 0.3 days to 0.58 days, while it ranged from 3.7 to 6.03 days in the second phase. Based on the half lives, the efficiency of the three bacterial species to degrade pendimethalin can be ordered as follows; *Bacillus mycoides* was more efficient than *Pseudomonas aeruginosa* which was more efficient than *Bacillus cereus*.

Key words: Bacteria; pendimethalin; biodegradation; Sudan

INTRODUCTION

Pendimethalin is the second main herbicide in the Sudan which constitutes about 7% of herbicide annual import after 2,-4-D (Abdelbagi and Mohamed 2006). It is registered in the Sudan under 17 trade names and used in the major irrigated agricultural schemes such as Gezira, Elrahad and New Halfa. This herbicide is applied as pre-emergence, pre-transplanting or early post-emergence herbicide for many crops including, cereals onions, garlic, maize, sorghum, rice, soya beans, peanuts, carrots, celery, peas, field beans, potatoes, cotton, citruses, lettuce, transplanted tomatoes and sunflower (Tomlin 2003). Pendimethalin is a dinitroaniline herbicide inhibiting plant cell division after germination and following emergence from the soil. U.S Environmental Protection Agency has classified pendimethalin as persistent bio-accumulative toxicant (USEPA 1999). The chemical has the ability to bio-accumulate, bio-magnify and can bio-concentrate up to 70,000 times its original concentration (Ritter *et al.* 2007). It is of low volatility, mobility in soil as well as low water solubility (Schleicher *et al.* 1995). Slight loss can occur from photodecomposition and volatilization. It is strongly adsorbed by moist soil, practically insoluble in water and thus does not leach appreciably in moist soil (Aktar *et al.* 2008). It contains dinitroanilines, which could result in the formation of the carcinogenic nitrosamines (USEPA 1985). Pendimethalin is highly toxic to fish and aquatic invertebrates (Meister 1992) and has been classified as possible human carcinogen (USEPA 1992). It disrupts the endocrine, reproductive, and immune systems and can cause neurobehavioral disorders (Ritter *et al.* 2007). Pendimethalin appears to be poorly absorbed and rapidly excreted. About 95% is excreted within 24 hrs after oral administration, 75% being found in the

feces and 20% in the urine. Maximum tissue concentrations were found in the liver and kidney. Although most of the parent compound is excreted unchanged, the metabolites identified suggested that oxidation of *3, 4*-methyl groups on the phenyl moiety and the *N*-alkyl side chain of the dinitro-substituted aniline are the predominant metabolic pathways (American Cyanamid 1986).

Strains of microorganisms isolated from pesticides polluted soils in Sudan were reported to have great capacity for the degradation of some pesticides such as lindane and endosulfan α and β isomers (Elsaid and Abdelbagi, 2010). Degradation capability of these strains can be enhanced by many activators such as farm manure and synthetic fertilizers (Elsaid *et al.* 2009). The potential use of indigenous soil microorganisms in cleaning pesticides polluted soils and dump sites was first argued by Abdelbagi *et al.* (2000; 2003). Considering the wide use of pendimethalin in the Sudan as soil applied herbicide and its persistent, bioaccumulation, biomagnification and bio-concentration properties (Ritter *et al.* 2007), as well as the promising potential of microorganisms isolated from pesticides polluted soil in degrading pesticides (Elsaid and Abdelbagi 2010), this study was initiated to cast light on the potential capability of some indigenous bacterial strains isolated from pesticides polluted soils viz; *Pseudomonas aeruginosa, Bacillus mycodies* and *Bacillus cereus* in degrading pendimethalin in mineral salt medium as well as to characterize the degradation models.

MATERIALS AND METHODS

Chemicals and reagents

Analytical standard of the herbicide, pendimethalin (99.9% pure) was obtained from Agricultural Research Corporation, Sudan. Acetone (99.8 pure), hexane (99.8 pure) and ethanol (99.8 pure) were obtained from Fischer Company, U.K.

Pendimethalin used for the biodegradation study (99% pure) was obtained by re-crystallization from a commercial pendimethalin sample (ESTOM 330gL^{-1}). The re-crystallization was done by dissolving 250 mL of commercial sample (ESTOM) in a minimum amount of solvent at room

temperature until over-saturation. The temperature was then raised to 60°C until the sample was dissolved. The sample was then cooled down to 4°C by placing the conical flask in an ice-water bath. The yellow pendimethalin crystals formed were then filtered through a filter paper and collected in a clean amber glass container. The re-crystallization was repeated three times and crystals were washed with acetone to increase the purity. The purity of the re-crystallized sample was checked by a GC equipped with a flame ionization detector against authentic analytical standard (purity 99.9%) obtained from ARC. The identity was confirmed by GC-MS.

Isolation and identification of microorganisms from pesticides polluted soils

Surface soil samples were randomly collected from pesticides-polluted storage soil at Hasahesa (Gezira scheme) using a soil auger of 10 cm depth and 5 cm diameter. Five auger-fulls were taken and mixed thoroughly to make a composite sample (one kg). The collected sample was placed in a paper bag, labeled and immediately transported to the pesticides laboratory.

Collected samples were immediately examined for microbial composition. Isolation and identification was done according to the methods described by Barrow and Feltham (2003). The identified bacterial strains were sub-cultured in meat peptone agar for 24 hrs prior to their use in biodegradation studies in carbon-free media.

Preparation of media

(i) Meat Peptone Agar (MPA): The MPA was prepared by adding 5g meat extract, 7.5g peptone, 5g NaCl and 20g agar to one liter distilled water according to the method of Tepper *et al.* (1993).

(ii) Mineral Salt Medium (MSM): The MSM was prepared following the method described by Tepper *et al.* (1993). 1g K_2HPO_4, 0.5 g $MgSO_4$.$7H_2O$, 0.5 g NaCl, 0.001g $FeSO_4.7H_2O$, 0.01g $MnSO_4.4$ H_2O, 0.05g $CaCO_3$ were added to a conical flask (1500 ml) and then, the volume was completed to one liter by adding distilled water.

The media were autoclaved for 20 minutes, at 121°C then allowed to cool at room temperature and kept in refrigerator at 4°C until used.

Preparation of the microbial inoculums

Three quantities of MPA, 200 ml each, were taken and each was placed separately in 250 ml conical flask. Each flask was inoculated with one bacterial strain using sterilized loops. Inoculated flasks were then closed with sterilized cotton and kept in an incubator (thermostatic cabinet) at 25 °C for 24 hrs for use in the biodegradation experiment.

Microbial degradation of pendimethalin in Mineral Salt Medium

The aim of this experiment was to evaluate the capability of the isolated and identified bacterial strains to degrade pendimethalin in Mineral Salt Medium.

A total of 60 clean test tubes were sterilized in an oven for three hours at 180°C. Ten mL of MSM was taken from the stock flasks into each test tube. One mL of inoculum was added to each test tube. The cultured test tubes were incubated at 25°C with 400 mgL^{-1} pendimethalin for 0, 3, 7, 15 and 30 days. The experimental units were arranged in a completely randomized design (CRD) with three replicates. Control sets without bacterial inocula were incubated under the same conditions. The recovery sets were immediately extracted and kept in the refrigerator for the gas chromatographic (GC) analysis.

Extraction of pendimethalin from the culture

Treated cultures were centrifuged at 800 rpm for 10 minutes to separate the microorganisms from the media. The supernatant was removed by careful decanting and placed in 100 ml separating funnel. Ten ml of hexane: acetone mixture (3: 1 respectively), were added followed by 5 mL saturated sodium chloride solution and one mL methanol. The contents were vigorously shaken for five minutes and allowed to stand for one minute until separation of layers. The hexane layer was collected in a clean test tube and the aqueous layer was re-extracted twice with 10 mL hexane. Hexane fractions were recombined in a clean test tube and dried up by passing through anhydrous sodium sulphate on filter paper. The

solvent was stripped off by rotary evaporator at 68°C till dryness, and the residues were re-constituted in 10 mL hexane and stored in the refrigerator at 4°C for GC analysis.

Gas chromatographic analysis

A gas chromatograph equipped with flame ionization detector (FID) and DB-5 fused silica capillary column of 30 m and 0.25 µm id, was used for analysis of the extracts. The stationary phase (0.25mm thickness) was 5% phenyl, methylpolysiloxane. Detector and injector temperatures were 300°C, 280°C and 230°C, respectively. Helium was used as a carrier gas at a flow rate of 4.23mL minute^{-1}. The oven temperature was programmed as follows: initial temperature was 50°C increased at 5°C minute^{-1} until 75°C, increased again at 10°C minute^{-1} until 160°C, increased by 5°C minute^{-1} until 180°C and finely increased by 3°C minute^{-1} until the final temperature of 240°C was reached, at which it was held for 2 minutes. Flow rates of the makeup gas (helium), hydrogen, and air were 30, 40 and 400 mL minute^{-1} respectively. Analysis of sample was done by triplicate injection of 2 micro liters. Three concentrations (0.5, 0.67 and 1.0 ppm) of the analytical standard of pendimethalin (99.9% pure) were injected under the same conditions and response was used for the construction of the standard curve. Re-analysis of the analytical standard was repeated every morning to check for the performance of the machine. Septum was changed when necessary. The Limit of Detection (LOD) of pendimethalin was 0.77 ppm. The recovery of the method ranged from 70% to 99% (Table 1).

Gas chromatography with mass spectroscopy (GC-Ms) analysis

Three representative samples were reanalyzed using a Shimadzu GC-Ms Qp2010 system (Japan) with an AOC-5000 auto sampler. The gas chromatograph was fitted with Rtx5-Ms capillary column 30 m x 0.25 mm id, 0.25 µm film thickness from Restek (U K). Helium (purity $\geq$ 99.999%) was used as a carrier gas at a flow rate of 1.33mL minute^{-1}. The splitless injection temperature was 200°C. The oven temperature was programmed from initial temperature of 100°C (held for three min) to 200 °C at 10°C/min, then increased by 3 °C per minute to 230°C (held for 5 min.) and finally increased by 5 °C per minute to 300 °C and held for 3

minutes. The mass spectrometer was operated with electron impact (E I) source in the scan mode. The electron energy was 70 e V, and the interface temperature was maintained at 280°C. the solvent delay was set to 2 min.

Statistical analysis

The data were analyzed using the analysis of variance (ANOVA). Probability of 0.05 or less was considered significant (SAS 2004).

Table1. Recoveries (%) of pendimethalin form the media inoculated with different strains at various intervals (days).

Media inoculated with	3	7	15	30
P. aeruginosa	97.25	95.25	83.5	86.25
B. mycoides	80.75	75.5	70.5	74.5
B. cereus	98	97	80.25	98.75

RESULTS

Three dominant types of bacterial strains were isolated from pesticide polluted soil, *Bacillus mycoides*; *Bacillus cereus* and *Pseudomonas aeruginosa*.

Biodegradation of pendimethalin after incubation with three strains of bacteria in Mineral Salt Medium (MSM)

The indigenous bacterial strains showed clear and variable capability in degrading pendimethalin in mineral salt medium. Data in Table 2 indicated that the concentration of pendimethalin declined with the increase in the incubation periods. In media inoculated with *Pseudomonas aeruginosa*, the initial concentration of pendimethalin (400 ppm) decreased to 302, 277, 119 and 77 ppm, after 3, 7, 15 and 30 days of incubation respectively, whereas in media inoculated with *Bacillus mycoides* pendimethalin concentration decreased to, 195, 185, 157 and 113 ppm, following the same order. On the other hand, the respective drop in the concentration of pendimethalin caused by *Bacillus cereus* was 180, 130, 122 and 79 ppm. Generally the rate of pendimethalin disappearance was slow from day 7 on. However, in *Bacillus cereus*

cultures the rate was slow from day 3 on. There were significant differences between the levels of pendimethalin at various time intervals (Table 2). Less than 20% of the initial concentration was recorded 30 days after incubation with *Pseudomonas aeruginosa* and *Bacillus cereus,* whereas 29% of the initial amount was found after 30 days of incubation with *Bacillus mycoides* (Table 2; Figure 1). Despite the significant drop in the starting material no metabolites were detected *in Bacillus* cultures, while only *N*-(1-ethylpropyl)-3-methyl-2, 6-diaminobenzine (Figures 2-7) was detected at low level in *P. aeruginosa* culture indicating the capability of these microorganismsfor complete mineralization of pendimethalin.

Biodegradation kinetics

A biphasic model was assumed in order to calculate the loss of pendimethalin from the media inoculated with different types of bacteria. Calculations were done according to the following equation:

$$R= A_0e^{-\alpha t}+B_0e^{-\beta t} \qquad \text{equation (1)}$$

Where R = amount of pendimethalin at t days; A_0 and B_0 are concentration of pendimethalin at t=o; α and β are the disappearance rate constants for first and second phase model respectively. The half-life of exponential decay was calculated according to equation 2.

$$t_{1/2} = (2.303\log 2)/\text{rate constant} \qquad \text{equation (2)}$$

The data in table 3 indicated that there was a faster rate of disappearance in the first phase than the second one. This is clearly reflected in the half-life values obtained. The half lives of the first phase were estimated at, 0.33, 0.39 and 0.58 days while the corresponding values for the second phase were; 3.64, 1.03 and 6.03 days in media inoculated with *Pseudomonas aeruginosa, Bacillus mycoides and Bacillus cereus*, respectively.

Table 2. Concentration of Pendimethalin (ppm) following incubation with the three of bacteria strains in mineral salt medium

Time (days)	*P. aeurginosa*	*B. mycoides*	*B. cereus*	LSD
	Mean ± S standard deviation			
0	$400^{a} \pm 0.0$	$400^{a} \pm 0.0$	$400^{a} \pm 0.0$	
3	$302^{b} \pm 0.023$	$195^{c} \pm 0.011$	$180^{c} \pm 0.029$	80.509
7	$277^{b} \pm 0.038$	$185^{c} \pm 0.02$	$130^{d} \pm 0.008$	69.309
15	$119^{c} \pm 0.005$	$157^{d} \pm 0.017$	$122^{b} \pm 0.003$	35.461
30	$77^{c} \pm 0.024$	$113^{d} \pm 0.004$	$79^{b} \pm 0.025$	58.181

Means followed with the same letter in the same raw are not significantly different at p= 0.05 according to LSD.

Table 3: Statistical parameters of pendimethalin bacterial dissipation in mineral salt medium

Statistical parameters	*P. aeruginosa*	*B. mycoides*	*B. cereus*
A_0	381	345	400
B_0	258	190	171
α ($days^{-1}$)	0.05	0.10	0.26
β ($days^{-1}$)	0.04	0.01	0.02
$t_{1/2\alpha\ (days)}$	0.33	0.39	0.58
$t_{1/2\beta\ (days)}$	3.64	1.03	6.03
Regression coefficient	0.915	0.847	0.979

A_0 and B_0 are the concentration of pendimethalin at t=0 and α and β are the disappearance rate constants for the first and second phase model, respectively.

DISCUSSION

The results showed that, *Pseudomonas aeruginosa, Bacillus mycoides* and *Bacillus cereus* isolated from pesticides polluted soil are capable of and quite efficient in degrading pendimethalin under the conditions of mineral salt medium. *Bacillus cereus* reduced the half life of pendimethalin to less than 0.6 days in the first phase ($t_{1/2\alpha}$) and less than 6.03 days in the second

phase ($t_{1/2\beta}$). The corresponding values for *Bacillus mycoides* and *Pseudomonas aeruginosa* were even shorter (0.39 and 1.03 days and 0.33 and 3.64 days respectively). This can be considered as a very significant reduction when compared to the reported half lives (30-90 days) of pendimethalin in soil. Pendimethalin was reported to be degraded in soil by many microorganisms such as bacteria {*Bacillus megaterium*, *Pseudomonas* sp., *Pyricularia* sp., *Rhizobium* sp., *Trichoderma viride* (Kole *et al*. 1994)} and fungi {*Fusarium oxysporum, Paecilomyces variotii*, *Rhizocotonia bataticola* (Singh and Kulshrestha 1991)}. Bacterial degradation of pendimethalin is dependant not only on culture conditions, but also on strains of bacteria present (Lee *et al*. 2004). *Bacillus cereus, Bacillus mycoides* and *Pseudomonas aeruginosa* were reported as degraders of many organic compounds such as petroleum products (Okerentugba and Ezeronye 2003).

The current study agrees with the argument of Abdelbagi *et al.* (2000; 2003) that indigenous soil microorganisms could be of great potential in reducing the level of contamination by pesticides in highly polluted storage soils in the Sudan. Their suggestion is in line with Elzorgani (1982) who mentioned that irrespective of the large amount of the DDT and other pesticides applied in Gezira scheme, Sudan, their soil level is not high which indicates possible and efficient degradation factors in these soils. This argument was later confirmed by Elsaid *et al.* (2009), who demonstrated the capability and efficiency of indigenous soil microorganisms (bacteria and fungi) in degrading endosulfan and lindane under conditions of selective mineral salt medium or soil. These strains can tolerate up to 1000 ppm of endosulfan without significant reduction in their degradation capability (Elsaid and Abdelbagi 2010). In addition, various types of synthetic and natural fertilizers enhanced the degradation rates (Elsaid *et al.* 2009).

B. mycoides and *P. aeruginosa* showed relatively higher performance in degrading pendimethalin. *P. aeruginosa* is versatile and previous reports suggested that it can degrade a number of chemicals including many pesticides such as carbanyl (Vandana and Phale 2005), malathion (Hashmi and Khan 2004) chloryprifos (Geetha and Fulekar 2008), *p*-

nitrophenol and parathion (Douglas and Dennis 1974). *P. aeruginosa* is a widely present gram negative soil bacterium and can be used to clean different man-made xenobiotic compounds (Geetha and Fulekar 2008). Its effectiveness in bioremediation under controlled environmental conditions was evaluated. Environmental factors such as pH and temperature were found to have effects on biodegradability of chlorpyrifos by test microorganism (USEPA 1997).

The results indicated that pendimethalin biodegradation by the three bacterial strains followed a biphasic model of an initial phase of a fast rate of disappearance followed by a second phase of a slow disappearance rate. The first phase lasted for 7 days in the case of *P. aeruginosa* and *B. mycoides* cultures, while it lasted for 3 days in *B. cereus.* This phenomenon of biphasic disappearance in soil is common in many pesticides (Khaled *et al*. 2008). The relative importance of the phases depends upon the availability of the pollutants, hydrophobicity and affinity for organic matter (Rigas *et al.* 2007).

Despite the significant drop in the starting material, no metabolites were detected *in Bacillus* cultures while only *N*-(1-ethylpropyl)-3-methyl-2,6-diaminobenzine (Figures 2-6) was detected at low level in *P aeruginosa* culture indicating the capability of these microorganisms in complete mineralization of pendimethalin to simple and common molecules such as CO_2, H_2O,…etc. The absence of detectable levels of breakdown products on pesticides bio-degradation studies involving bacteria and fungi was reported by many authors (Khaled *et al*. 2008). The metabolic product *N*-(1-ethylpropyl)-3-methyl-2, 6-diaminobenzine detected in culture of *P. aeruginosa* could be formed by reduction of NO_2 groups into NH_2 and demethylation of one of the methyl groups. These results are in line with the findings of Singh and Kulshrestha (1991) who found that *Bacillus* sp. strain MS202 initially reduced the NO_2 group to NH_2 under aerobic conditions.

The current results indicated the great potential of indigenous microorganisms isolated from pesticide polluted soils in degrading pendimethalin under the conditions of mineral salt medium. In light of

this and previous results (Elsaid and Abdelbagi 2010; Elsaid *et al.* 2009), the use of such microorganisms in cleaning pesticides polluted soils through bioremediation techniques can be argued and deserves further investigation.

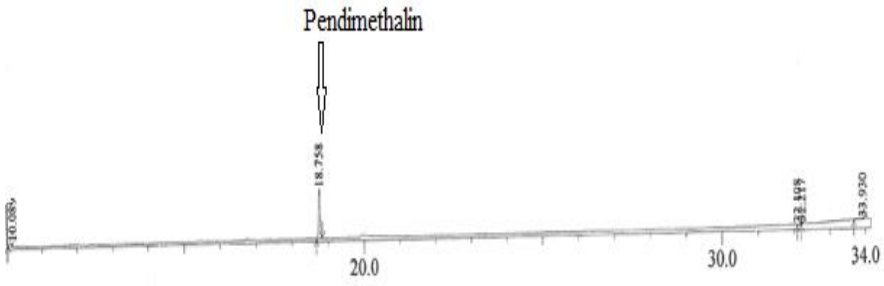

Figure 1. Typical chromatogram (Total Ion Current, TIC) of pendimethalin after 30 days of incubation with *Bacillus mycoides* in mineral salt medium (MSM)

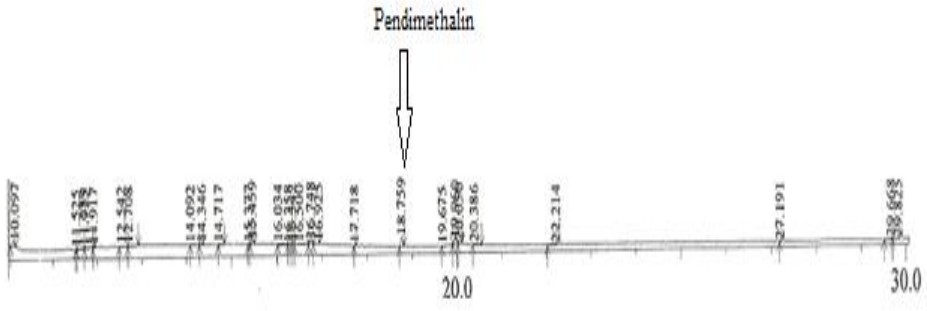

Figure 2. Typical chromatogram (TIC) of pendimethalin after 30 days of incubation with *Bacillus cerceus* in MSM

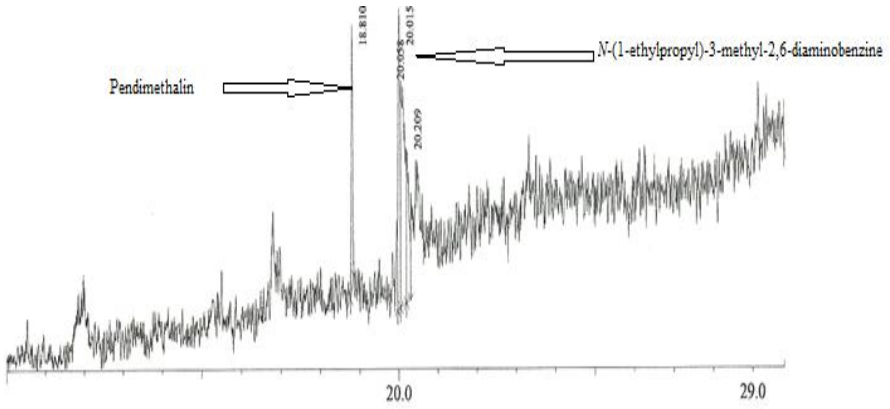

Figure 3. Typical chromatogram (TIC) of pendimethalin after 30 days of incubation with *Pseudomonas aeruginosa* in MSM

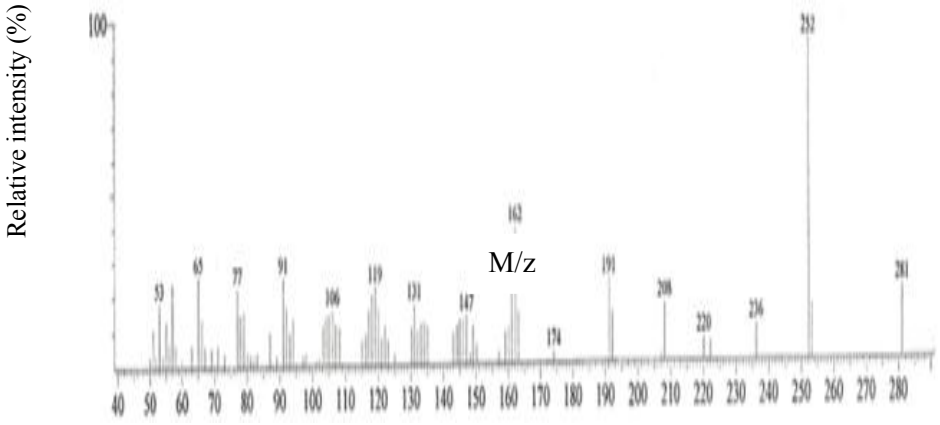

Figure 4. Mass spectrum of pendimethalin

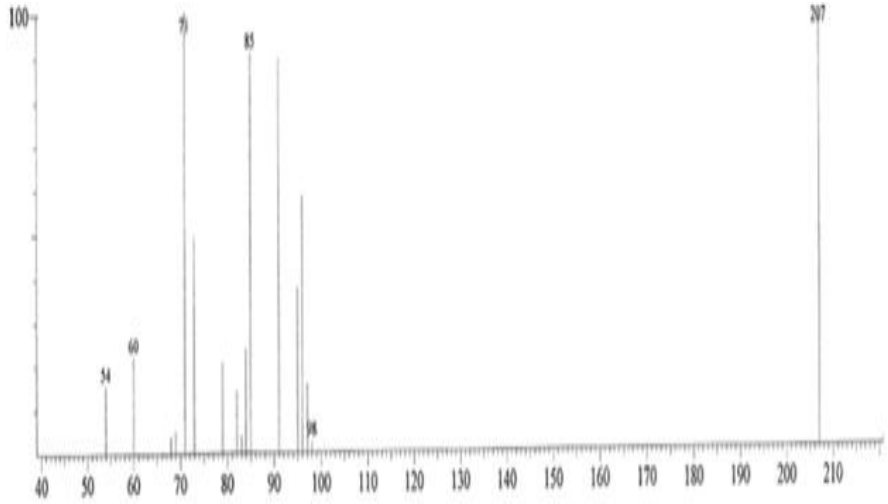

Figure 5. Mass spectrum of *N*-(1-ethylpropyl)-3-methyl-2, 6-diaminobenzine

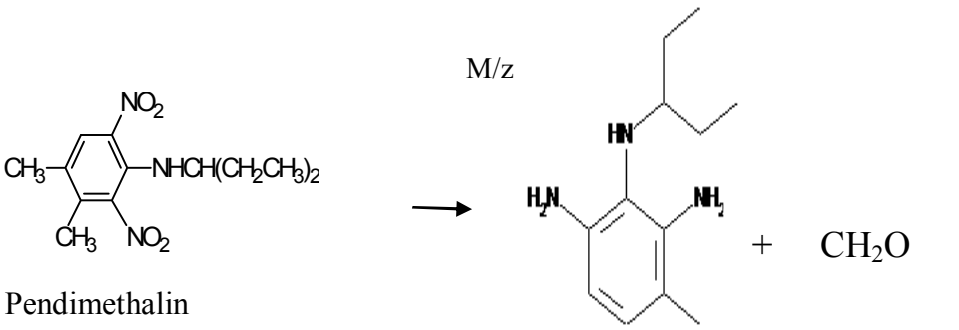

Figure 6. Proposed pass way of pendimethalin biodegradation by *Pseudomonas aeruginosa* in MSM

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Adam Ali Mohamed and Mr. Salah of the National Chemical Laboratories, Federal Ministry of Health, Sudan for their help with GC-MS analysis. The financial support made available by the Ministry of Higher Education and Scientific Research, Sudan is highly acknowledged.

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التحلل الحيوي لمبيد بنداميثالين بثلاثة سلالات من البكتيريا معزولة من تربة ملوثة بالمبيدات

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المستخلص: تمت دراسة التحلل الحيوي لمبيد بنداميثالين بثلاثة سلالات من البكتيريا (*Pseudomonas aeruginosa, Bacillus mycoides*, *Bacillus cereus*) عزلت من تربة ملوثة بالمبيدات في السودان. اجريت هذه الدراسة فى وسط غذائي خالي من الكربون. تم تحضين البنداميثالين مع البكتريا لمدة 0 و 3 و 7 و 15 و 30 يوم مع سحب عينات فى الفترات المحددة وتحليلها في جهاز الكروماتوجراف الغازى . تم حساب الفقد في التركيز الأبتدائي (400 جزء فى المليون) عبر فترة التحضين و استخدم لحساب نصف عمر المبيد بإتباع نموذج الاختفاء الثنائي. تم استخدام جهاز GC-MS للتعرف على النواتج الايضية والمادة الابتدائية (البنداميثالين). أظهرت النتائج أن النسب المئوية للكمية المتبقية من البنداميثالين والتى حضنت في وسط غذائي ملقح ببكتريا *Pseudomonas aeruginosa* كانت 75.5% و 69.25% و 29.75% و 19.25% بعد 3 و 7 و 15 و 30 يوم من التحضين علي التوالي . بينما في الوسط الغذائي الملقح ببكتريا *Bacillus mycoides* كانت النسب؛ 48.75% و 46.25% و 39.25% و 28.25% بنفس الترتيب ، من ناحية اخرى احدث التلقيح ب *Bacillus cereus* تناقص في كمية

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المبيد حيث بلغ المتبقي منه 45% و 32.5% و 30.5% و 19.75% بعد 3 و7 و15 و30 يوم على التوالي . علي الرغم من النقص أو الفقد الكبير في المادة الابتدائية إلا أنه لم يتم التعرف على اى نواتج ايضية فى الاوساط الملقحة ببكتريا *Bacillus* بينما تم التعرف على ناتج أيضى واحد *N*-(1-ethylpropyl)-3-methyl-2,6-diaminobenzine فى الوسط الملقح ب *P. aeruginosa* مما يدل على مقدرة هذه الكائنات على احداث تمعدن كامل (Mineralization) للبنداميثالين. وجد أن التحلل الحيوي للبنداميثالين بالسلالات الثلاثة من البكتريا يتبع نموذج الاختفاء الثنائى حيث كان معدل الاختفاء فى المرحلة الأولى أسرع من المرحلة الثانية . تراوح نصف عمر المبيد فى المرحلة الأولى من 0.3 يوم إلى 0.58 يوم مقارنة بـــ 3.7 يوم إلي 6.03 يوم في المرحلة الثانية . بناءاً عليه يمكن ترتيب كفاءة الكائنات الثلاثة كما يلي؛

Bacillus mycoides أفضل من *Pseudomonas aeruginosa* أفضل من *Bacillus cereus* .