J. Plant Prot. and Path., Mansoura Univ., Vol. ٤ (٥): ٤٦٣- ٤٧٢, ٢٠١٣

DISSIPATION OF PENDIMETHALIN BY *Bacillus megaterium*

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ABSTRACT

One strain of microorganisms was isolated from soil previously treated with pendimethalin using enrichment technique and identified as *Bacillus megaterium* (E٢٢). The effect of pH and temperature on the growth ability of the tested strain was investigated. The results showed that the optimum pH and temperature for the growth of pendimethalin degrading strain were ٧ and ٣٠ °C, respectively. Some concentration of pendimethalin was removed by *B. megaterium* remove from mineral liquid medium with half-life of ٥.٦ days. Pendimethalin half-life was ٥٣.٤ days in untreated mineral liquid medium as control. Also, there is no toxicity of pendimethalin detected on *Rhizobium leguminsarum* biovar viciae as test microorganism in the supernatant after ٢٨ day of treatment with *B. megaterium*. The results suggest that bioremediation by *B. megaterium* isolate was considered to be effective method for detoxification of pendimethalin herbicidein aqueous media.

Keyword: Pendimethalin, Soil, Bioremediation, phytotoxicity, seedling mortality.

INTRODUCTION

Pendimethalin,N-(١-ethylpropyl)-٢,٦-dinitro-٣،٤-xylidine,had empirical formula $C_{١٣}H_{١٩}N_{٣}O_{٤}$, a selective preemergent herbicide in dinitroaniline group was used extensively for weed control in cotton, rice, soybean and tobacco (Smith *et al*., ١٩٩٥). Pendimethalin acts by inhibiting the steps in plant cell division responsible for chromosome separation and cell wall formation. It is used before crop emergence or planting (Appleby and Valverde, ١٩٨٨). The inhibition of root and shoot growth results in stunting of aerial plant Portions (Parka and Soper, ١٩٧٧). Studies in terrestrial ecosystems showed that ١٠–٢٠٪ of the herbicide vaporizes within the first week or two weeks after application (Strandberg and Scott-Fordsmand(٢٠٠٤).Care should be taken to minimize excessive pendimethalin applications to the soil in order to minimize possible injury to sensitive rotation crops. It is important to develop the methodology to prevent pesticide contamination from. Microorganisms can use a variety of xenobiotic compounds including pesticides for their growth, mineralize and detoxify them (Belal *e tal*., ٢٠٠٨). Bioremediation is an accepted technology for accelerating the rate of cleanup of contaminated water and soil. Soil microorganisms that are repeatedly exposed to pesticides may develop new capabilities to degrade such chemicals (Vidali, ٢٠٠١). There are some reports on the degradation of pendimethalin by microorganisms comprising *Azotobacter chroococcum*, *A. vinelandii* and *Bacillus circulans* (Saha *et al*., ١٩٩١; Singh and Kulashrestha, ١٩٩١; Kole *et al*., ١٩٩٤; Megadi *et al*., ٢٠١٠). The success of bioremediation depends not only on the high degradation ability but also on the stability of active microorganisms under varied conditions, such as

changes in pH and temperature (Pattanasupong *et al*., ٢٠٠٤). Therefore, it is necessary to investigate the effects of various environmental factors on the growth ability of the tested microorganisms (Pattanasupong *et al*., ٢٠٠٤). However, after remediation toxicity assessments are needed. Firstly, is providing valuable and complementary information to compound analysis. Secondly, the major advantage of toxicity tests is the direct assessment of the potential hazard to the environmental system by both original pollutants and its metabolites (Tiainen *et al*., ٢٠٠٢). Therefore, this study attempted to isolate and identify efficient bacterial strain for bioremediation of pendimethalin in aquatic. In addition to confirm the complete detoxification of pendimethalin by measuring the toxicity of the aqueous medium in the presence of bacterial strain against sensitive target such as *Rhizobium leguminosarum* biovar *viciae*.

MATERIALS AND METHODS

Chemicals

Pendimethalin (N-(١-ethylpropyl)-٢،٦-dinitro-٣،٤-xylidine) standard was obtained from Ehrenstorfer (Germany). All other chemicals were of analytical grade.

Microbial degradation of the pendimethalin :

Media

M٩-Minimal medium as mineral salt liquid (MSL) and Luria Bertani (LB) a complete medium were used through this study as described by Sambrook *et al*. (١٩٨٩).

Isolation by enrichment culture

Enrichment cultures of microorganisms capable of degrading pendimethalin were established from soil that previously treated with pendimethalin. Samples of soils were collected from Kafr El-Sheikh and Elbeheira, Governorates, Egypt. Ten grams soil were suspended in ٩٠ ml sterilized mineral salt medium in ٥٠٠-ml bottle containing (١٠٠ µg/ml) of pendimethalin as a sole source of carbon, then incubated at ٣٠ °C and ١٥٠ r/min for ٢٨ days. Thereafter, ١٠ ml of the cultures were transferred into fresh ٩٠ ml MSL medium containing the same concentration of pendimethalin. This procedure was repeated four times. Series dilutions were prepared after the final time from enrichment culture in a glass tube containing ٩ ml MSL medium up to ١:١٠$^{-٦}$ and then ١٠٠ µl of it was speeded on plates of MSL medium + pendimethalin (١٠٠ µg/ml) using Drigalisky triangle. The plates were sealed in polyethylene bags, then incubated at ٣٠ °C for ٧ days and monitored for appearance of colonies. Single colony growing on each plate was isolated by picking the colony using sterile inoculating needle and was further purified by the standard spatial streaking on complex agar media for bacterial isolate. The isolated colonies were then tested for their ability to grow in MSL medium containing (١٠٠ µg/ml) of pendimethalin. The number of cells of each strain was determined by plating appropriate dilutions of liquid medium onto mineral salt agar medium containing pendimethalin. Bacterial populations were estimated by counting the number of colonies on plates (Belal and El-Nady ٢٠١٣).

Identification

The efficient selected pendimethalin degrading bacterial isolate was identified depending morphological and physiological characteristics as described by to Parry *et al.,* (١٩٨٣).

Effect of temperature and pH on the growth of the tested strain:

To determine the effect of temperature and pH on the growth of tested strain, a ٣٠ ml MSL medium supplemented with ١٠٠ µg/ml of pendimethalin as a sole source of carbon for bacterial strain. MSL medium was inoculated by one ml from bacterial cell suspension at $١٠^{٧}$ cfu/ ml. To determine the optimum pH, experiments were carried out at pH ٦, ٦,٥, ٧, ٧,٥ and ٨. Cultures were incubated on a rotary shaker at ٣٠ °C and ١٥٠ r/ min for ٧ days. To determine the effect of temperature, MSL medium with pH of ٧ was incubated at ٢٠, ٢٥, ٣٠, ٣٥ and ٤٠ °C under ١٥٠ r/min. Cells number of the bacterial strain were determined by plating appropriate dilutions of liquid medium onto mineral salt agar medium containing pendimethalin.

Biodegradation of pendimethalin by *Bacillus megaterium* (E٢٢) in liquid medium:

Bacillus megaterium (E٢٢) was cultured onto MSL medium + pendimethalin for ٧ days and then the growing colonies were washed with ٣ ml sterilized MSL medium. The bacterial cell suspension ($١٠^{٧}$ cfu/ml) was then used to inoculate ١٠٠ ml MSL medium containing (١٠٠ µg/ml) of pendimethalin. The cultures were incubated at ٣٠ °C and ١٥٠ r/ min for ٠, ٧, ١٤, ٢١ and ٢٨ days. The percentage of degradation and the half-live of pendimethalin were determined as described afterward. Control flasks of equal volume of liquid mineral medium and pendimethalin without any microbial population were run in parallel at all intervals to assess a biotic loss.

Analytical procedure :

Extraction and determination of pendimethalin residues was carried out by the described method by Jaźwa *et al*. (٢٠٠٩) at Central Agric. Pesticides Laboratory, Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Egypt. The analysis of the samples was performed using a Hewlett Packard ٥٨٩٠A gas chromatograph, equipped with nitrogen – phosphorus detector (GC-NPD). The column used in this study was an HP fused – silica capillary column coated with cross-linked methyl silicone (length ٢٥ m, ID ٠,٣١, film thickness ٠,٥٢ µm). Nitrogen was used as both the carrier and make-up gas at a flow rate of ٣٠ ml/min. Hydrogen was used at a flaw rate of ٣,٥ ml/min. and air at ١٢٠ ml/min. The oven temperature was programmed as follows: initial temperature ١٥٠ °C (١ min.), rate of ١٠ °C/min. and final temperature ٢٥٠ °C. Recovery studies were carried out regularly by spiking analytical samples with stock solution of pendimethalin standard.

Totoxicity test:

The toxicity bioassay of pendimethalin was performed on the aqueous solutions after ٢٨ days after treatment with the tested microbial isolates. *Rhizobium leguminosarum* biovar *viciae*, as ae a test microorganism to pendimethalin was used as the test organism. The toxicity was determined as percentage of inhibition in the growth of tested bacteria comparing to control treatment. Yeast extract manitol agar medium was poured into Petri

dishes (٩ cm in diameter, ١٥ ml /dish), after solidification, wells were punched in each plate. The plates were inoculated by spreading ١٠٠ µl (١٠^{٨} cfu/ ml) from growth suspension of *Rhizobium leguminosarum* biovar *viciae* on Yeast extract manitol agar plates. After that, ٥٠ µl from the supernatant was put in punched holes (٠,٥mm in diameter) in Yeast extract manitol agar plates, where the culture broth was obtained by culturing of the pendimethalin degrading isolate with pendimethalin in MSL medium after ٢٨ days. The culture broth was filtrated through a sterile membrane filter (٠,٢ ìm). On the other hand, ٥٠ µl from sterilized liquid medium was put in punched holes which were used as control treatments. Experiments were made in three replicates. Plates were incubates at ٢٨°C. Diameter of inhibition zone (mm) surrounding each hole was recorded after ٧٢hrs.

RESULTS AND DISCUSSION

Isolation of the pendimethalin-degrading isolates:

The pre-treated soil samples with pendimethalin were used to isolate the pendimethalin-degrading microorganisms in the present study. By using enrichment techniques,. A preliminary classification based on the morphology of the isolates revealed that, the pendimethalin-degrading microorganisms belongs to the group of bacteria. The isolate was Gram positive, motile, spore forming rod shaped bacterium. This bacterial strain (E٢٢) was identified according to morphological, physiological chacterstics as *Bacillus megaterium*.

Our results are in agreement with previous finding reported by (Kopytko *et al*., ٢٠٠٢; Karpouzas *et al*., ٢٠٠٥; Belal *et al*., ٢٠٠٨; Megadi *et al*., ٢٠١٠). It was found that enrichment culture technique led to the isolation of two bacterial strains, which were able to degrade different pesticides rapidly in liquid cultures. Chaudhry and Ali, ١٩٨٨ reported that, the application of pendimethalin promotes the evolution of microorganisms that are capable of degrading this xenobiotic compound in the soil.

Also, Chaudhry and Ali, (١٩٨٨) reported that, actinomycetes have considerable potential for the biotransformation and biodegradation of pesticides. On the other hand members of this group were gram-positive bacteria and have been found to degrade pesticides with widely different chemical structures including organochlorines, *s*triazines, triazinones, carbamates, organophosphates, organophosphonate, acetanilides, sulfonylureas and herbicide,metolachlor (Krause *et al*., ١٩٨٥; De Schrijver and De-Mot, ١٩٩٩).

Effect of environmental factors on the growth of *Bacillus megaterium* (E٢٢) :

Effect of pH

Normally, the pH and temperature influence the growth of microorganisms and hence, these factors will influence also the degradation process of the pesticides. Karpouzas and Walker (٢٠٠٠) reported that the degradation of ethoprophos by *P. putida* strains epI and II affected by pH and temperature. Hong *et al*. (٢٠٠٧) found that various factors including pH and temperature affected degradation of fenitrothion-contaminated soil using

Burkholderia sp. FDS-١. Belal *et al*. (٢٠٠٨) found also that the pH and temperature affected cadusafos, carbofuran and cymoxanil degrading microorganisms comprising fungi and bacteria. The influence of pH on biomass yield by the tested isolates is shown in Fig. ١.

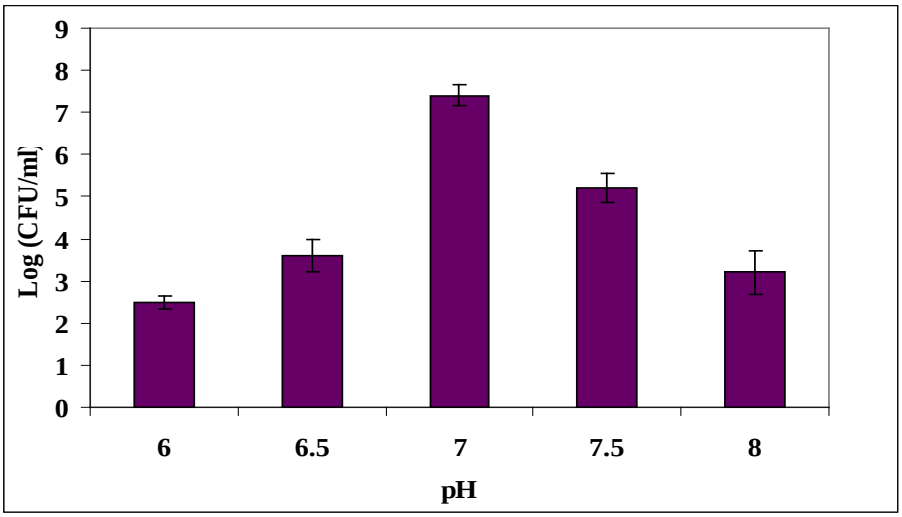

Figure ١: Effect of pH on the growth ability of *Bacillus megaterium* (E٢٢).

Generally, the optimum pH was ٧ for either bacterial or fungal isolates. Since, the maximum intracellular protein content for *Bacillus megaterium* (E٢٢) were recorded at pH ٧ (Fig. ١). It is known that the most of bacterial isolates prefer the neutral pH. However, the tested bacterial strain in this study can grow at range of pH from ٦ to ٨.

Ross and Marco, (١٩٧٨) reported previously that metalaxyl acid (N-(٢،٦-dimethylphenyl)-N-(methoxyacetyl)-alanine) resulting from the hydrolysis of the methyl ester group of pendimethalin which determined as the major metabolite in field soils at potato harvest and may this allow to effect degrading of metalaxyl.

Effect of temperature

The effect of different temperatures on the growth of *P. putida* (E٢٢) is shown in Fig. ٢. The temperature of ٣٠ °C appears to be the optimum degree for growth of *Bacillus megaterium* (E٢٢). Moreover, the tested microbial isolates exhibited growth at ٤٠ °C. of *Bacillus megaterium* (E٢٢)was used for further studies under the optimum growth conditions to evaluate their degradation potential for pendimethalin at different incubation times (٠, ٧, ١٤, ٢١, ٢٨ days).

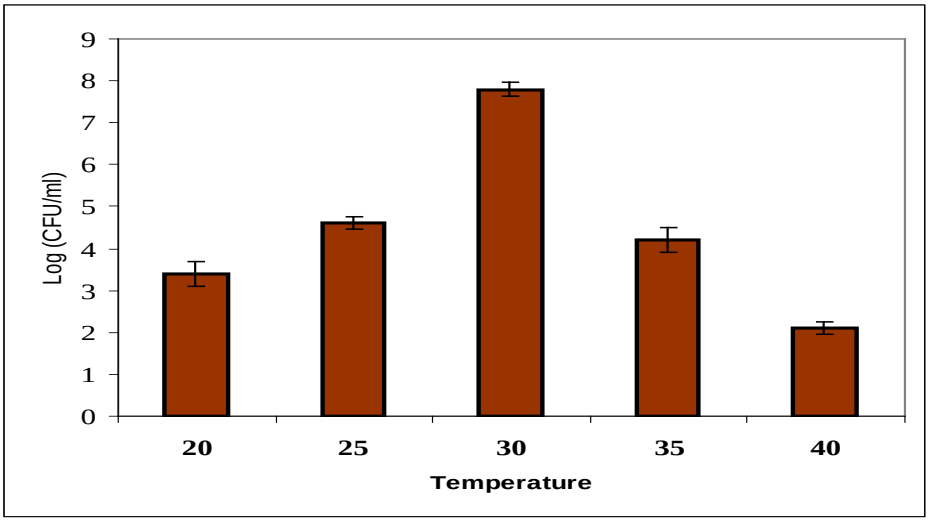

Figure ٢: Effect of temperature on the growth ability of *Bacillus megaterium* (E٢٢).

Biodegradation of pendimethalin by *P. putida* (E١٥) in aquatic system

The ability of *Bacillus megaterium* (E٢٢) to degrade pendimethalin was illustrated in Fig. ٣. The results indicate that *Bacillus megaterium* (E٢٢) was most efficient strain in pendimethalin degradation with a half-live of ٥. ٦ days. One hundred percent of pendimethalin initial concentration was degraded within ٤ weeks by *P. putida* (E١٥). Pendimethalin half-live was ٥٣.٤ days in untreated liquid medium as control treatment.

The growth response of pendimethalin *Bacillus megaterium* (E٢٢) was increased gradually with the pendimethalin degradation rate increasing as shown in Fig. ٣.

This is suggesting that different microbial types, which may be using different enzymes, have different degradation preferences. On the other hand, pendimethalin degradation percentage reached to ١٧٪ at the end of incubation time in control or non-inoculated samples. This implies that the quote of pendimethalin decay due to temperature effect and volatilization (Strandberg and Scott-Fordsmand, ٢٠٠٤). Many authors reported earlier that *Pseudomonas* has considerable potential for the biotransformation and biodegradation of pesticides. Members of this group are gram-negative bacteria and have been found to degrade pesticides with widely different chemical structures (Spain and Nishino, ١٩٨٧; Kyria *et al*., ١٩٩٧). The degradation of some pesticides may be attributed to the secretion of enzymes from either tested bacterial or fungal strains which are capable of degrading of pesticides (Bollag and Liu, ١٩٩٠).

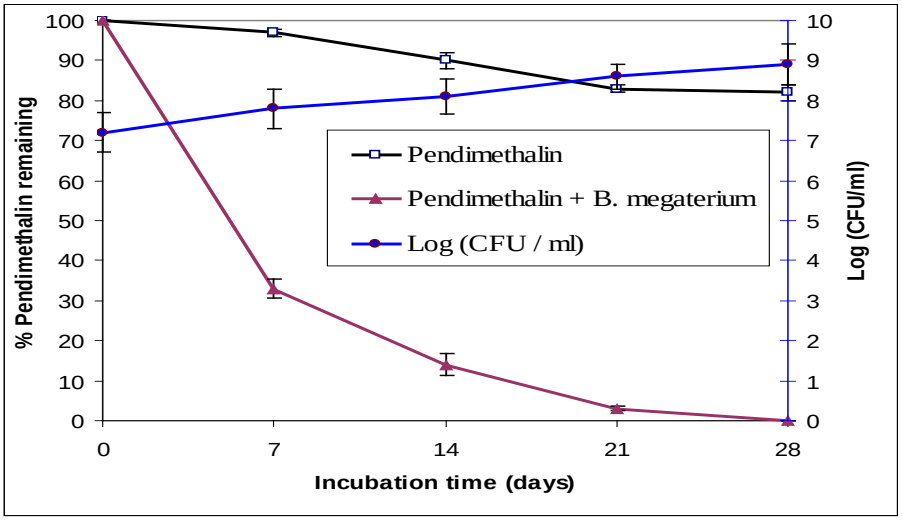

Figure ٣: Biodegradation of pendimethalin by *Bacillus megaterium* (E٢٢) in aquatic system.

With regard to biological metabolization, in vitro degradation of pendimethalin has been demonstrated by numerous authors. For instance, Kole *et al*., (١٩٩٤) observed that ٤٥٪ and ٥٥٪ metabolism of pendimethalin caused by *Azotobacter chroococcum* after ١٠ and ٢٠ days of incubation, respectively. *Azotobacter vinelandii* was isolated from a pendimethalin-treated barley rhizosphere. *A. vinelandii* utilized pendimethalin as the sole source of carbon to fix N_2 (Saha *et al*., ١٩٩١; Singh and Kulashrestha, ١٩٩١). Pendimethalin was degraded by oxidative N-dealkylation to yield ٣،٤-dimethyl-٢،٦-dinitroaniline and pentane. However, ٦-aminopenimethalin and ٣،٤-dimethyl-٢،٦ dinitroaniline were not further metabolized because they neither supported growth of organism nor stimulated oxygen uptake. But the pentane, released by oxidative N-dealkylation of pendimethalin, was utilized as the sole source of carbon and energy for the growth of the organism. The acetylation, aryl methyl oxidation and cyclization products of pendimethalin, as reported in *Azotobacter chroococcum* (Holding and Collee, ١٩٧١) were not detected in culture filtrate of *Bacillus circulans* (Kole *et al*., ١٩٩٤). Megadi et al. (٢٠١٠) reported that pendimethalin degradation with *Bacillus circulans* was by nitroreduction and oxidative N-dealkylation via secretion of pendimethalin nitroreductase and pendimethalin N-dealkylase. The microbial degradation of dinitroaniline herbicides, pendimethalin and trifluralin has been reported to occur most often by oxidative N-dealkylation and nitroreduction. The nitro group reduction and oxidative N-dealkylation destroys the herbicidal activity of pendimethalin, leading to its detoxification (Zayed *et al*., ١٩٨٣; Singh and Kulashrestha, ١٩٩١; Kole *et al*., ١٩٩٤; Megadi *et al*., ٢٠١٠).

Toxicity assessment

The remaining toxicity of pendimethalin against *Rhizobium leguminosarum* biovar *viciae* in the aqueous solutions after ٢٨ days after

treatment with tested microbial isolates was estimated in Table ١. The results show that the supernatant of pendimethalin after ٢٨ d treatment with *Bacillus meaterium* (E٢٢) had no antibacterial activity when bioassay was conducted with *Rhizobium leguminosarum* biovar *viciae* as a test organism. The obtained results were compared with control treatment. This implies that the aqueous solution spiked with pendimethalin was completely detoxified after ٢٨ days of treatment with *Bacillus meaterium* (E٢٢) isolate. The bioassay test also reassures the aforementioned obtained results with analysis by using gas chromatograph. Our results are agree with the finding of Bailey and Coffey (١٩٨٦) who used the same

Table ١: Toxicity of pendimethalin solutions against *Rhizobium leguminosarum* biovar *viciae* after ٢٨ days incubation with *Bacillus meaterium* (E٢٢).

Treatments	% Inhibition
Bacillus meaterium (E٢٢)	٠
pendimethalin	١٠٠

Conclusion

In the present study, bioremediation of pendimethalin-contaminated aqueous medium was studied by addition of pure culture from *Bacillus meaterium* (E٢٢) in ٢٨ days. *Bacillus meaterium* (E٢٢) showed high ability in Pendimethalin degradation. There is no toxicity of pendimethalin detected in aqueous medium after it treated with *Bacillus meaterium* (E٢٢) on *Rhizobium leguminosarum* biovar *viciae*. The results suggest that bioremediation by *Bacillus meaterium* (E٢٢) strain and compost were considered to be the effective method for detoxification of pendimethalin in aqaeous system.

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ازالــة مبيــد الحشــائش بينــداميثالين بواســطة البكتريــا العصــوية *Bacillus megaterium*

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تم عزل و التعرف عاى احد سلالات الكائنات الحية الدقيقة وهى نوع من البكتريا العصوية *Bacillus megaterium* (E٢٢) من التربة التى سبق معاملتها بمبيد الحشائش بيندامیثيالين باستخدام تقنية enrichment technique . وعند دراسة تأثير درجة الحموضة ودرجة الحرارة على قدرة نمو السلالة المختبر، أظهرت النتائج أن درجة الحموضة ودرجة الحرارة المثلى لنمو هذه السلالة التى لها االقدرة على تحطيم مبيد بيندامیثالين هى ٧ و ٣٠ درجة مئوية على التوالي. واستخدمت هذه البكتريا فى ازالة مبيد بيندامیثالين من البيئة السائلة المعدنية و كانت فترة عمر نصف مبيد بيندامیثالين ٥،٦ يوما بينما كانت فى الكنترول ٥٣،٤ يوما فى البيئة السائلة المعدنية الغير معالجة. ولا يوجد أي سمية لمبيد بيندامیثالين على بكتريا العقد الجذرية فى الفول Rhizobium leguminsarum بواسطة اختبارالكائنات الحية الدقيقة فى المحلول بعد ٢٨ يوما من معاملتها بالبكتريا *B. megaterium* . وتشير النتائج إلى أن المعالجة البيولوجية بواسطة بكتريا *B. megaterium* التى تم عزلها تعتبر طريقة فعالة لإزالة سمية مبيد بيندامیثالين من البيئة المائية.

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