BACILLUS MEGATERIUM BIODEGRADATION GLYCOPHATE

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ABSTRACT

This study was aimed to evaluate the *Bacillus megatrium* ability to growth and degradated the organophosphorus pesticides, Glyphosate. , *Bacillus megaterium* was isolated from Iraqi Soils and identification by morphological and biochemical tests beside a Sperber's Medium as selectivity media. The best growth results were in (2- 60) days, had the same growth for both (5, 25) ppm on MSM. The best degradation rate ability % were in (25) ppm /60 days (70.9)%. The increasing in incubation show increasing of degradation ration% of Glyphosate *via* HPLC specially after 60 days , the best ration were for (25)ppm .The result is the *B. megaterium* used the Glyphosate as source for carbon and phosphorus and suggest could be well exploited for bioremediation of Glyphosate contaminated sites.

Keywords : Organophosphorus-pesticides, Bacteria, Bio-remediation.

مجلة العلوم الزراعية العراقية -2019 :50(6):1674-1680 **موسى وآخرون**

التحلل الحيوي للكلايفوسفيت بواسطة *Bacillus megaterium*

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المستخلص

هدفت الدراسة الى تقيم فعالية *Bacillus megaterium* للنمو والتحطيم لاحد المبيدات العضوية الفسفورية، الكلافوسيت ، حيث تم عزل البكتريا من ترب العراق المحلية واجراء اختبارات الكشف المظهري والحيوي الكيايوي على الوسط الزرعي الانتخابي Sperber's Medium. كانت النتائج الافضل لنمو البكتريا في (2–60) يوم ولها نفس النمو على التراكيز ppn (5–25) في وسط الاملاح المعدنية MSM. افضل نسبة لقابلية النحليل المئوية % كانت عند تركيز 25ppm / 60 يوم (70.9)%. ان الزيادة في فترة الحضن اظهرت زيادة في نسبة التحلل % للكلايفوسيت من خلال تحليل جهاز كروموتوغرافيا السائل العالي الاداء خصوصا بعد 60 يوم، النسبة الافضل كانت عند تركيز (25)ppm. النتيجة كانت ان بكتريا *Bacillus megaterium* استخدمت الكلايفوسيت كمصدر كاربوني وفسفوري للنمو وممكن استغلالها للتحلل الحيوي للمواقع الملوثة بكلايفوسيت.

الكلمات المفتاحية: مبيدات الفسفور العضوية، البكتريا، التحلل الحيوي.

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INTRODUCTION

Organophosphate pesticides are heterogeneous compounds, containing a phosphoric acid derivative. ;Glyphosate is one of an organophosphate and non-selective herbicide , applied to the leaves of plants for killing both broadleaf plants and grasses .It was first registered for use in the U.S. in 1974 by Monsanto (Roundup) (18). Glyphosate stops a specific enzyme pathway, the shikimic acid pathway. The shikimic acid pathway is necessary for plants and some microorganisms. Beside the benefits of Using chemicals in pests control, they can cause potential human and environmental in case of extensive use (9). The genotoxicity and carcinogenicity studies for glyphosate and its commercial products (Roundup) were assessed. There was no convincing evidence for direct DNA damage in vitro or in vivo, and it was concluded that Roundup and its components do not pose a risk for various types of cancer in humans1.Glyphosate is difficult herbicide in trace analysis, has low molecular weight, low volatility, thermal lability, and good water solubility. These properties cause problems in extraction, purification and determination (9). The ability of Microorganism to remove pollutants from contaminated sites is one of promising treatment method (7).As an alternative strategy , is supported because of their effectiveness, minimize hazardous , economic value and environmental safety is known bioremediation (5) .Many researcher improved that different bacteria groups shown great ability of degradation Organophosphorus insecticides and others (3,6,15,19). The microorganisms strategies in degradation reaction towards pesticides in soils and they are co- metabolism, catabolism and metabolic enzymes (4). To determine the fate for pesticides in environmental , the microbial degradation can be a base factor for that., the study aim to carried out to investigate the ability of local bacterial isolated to tolerate and degrade Glyphosate in different concentrations and value the residue of it in extraction solution from media by HPLC.

MATERIAL AND METHODS

Chemical and reagents

Commercial pesticide "Glyphosate "was purchase from Iraqi market and other chemicals and reagents were in laboratories of Water and Environmental Directorate of Iraqi Ministry of Science and Technology. The media that used in growing *B. megaterium* to examine Glyphosate degradation was Mineral Salt Media(MSM) (0.2 g KH_2PO_4; 0.5 g K_2HPO_4 (sterilized separately at 125 ºC for 25 min to prevent precipitation and later aseptically added to the rest of the salts); 1g $(NH_4)_2SO_4$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.2 g NaCl; 0.05 g $CaCl_2 \cdot 2H_2O$; 0.025 g $FeSO_4 \cdot 7H_2O$; 0.005 g Na_2MoO_4; 0.0005 g $MnSO_4$ (pH 7.0 ± 0.3) (10). Flasks(125mL)were supplemented with Glph (Glyphosate) as the only carbon source. The Final Concentration of Glph were (5,10,15,20,25ppm) with 0.5 ml from inoculum bacteria in comparative with control.

Soil samples collection

Samples were taken from the top 15 cm of soil and kept in plastic bags at 4○C until use. Different samples of soil were collected treated and non-treated with organophosphorus pesticides and used for isolation microorganism by dilution (7).

Isolation and identification of *Bacillus megaterium* from soil

Bacillus megaterium was isolated by Sperber's Medium which is a selective medium for isolating it. The Sperber Media consist of :(Glucose - 10 g Yeast extract - 0.5 g $MgSO_4$. $7H_2O$ - 0.25 g $CaCl_2$ - 0.1 g Agar - 15 g Distilled water - 1000 ml , pH - 7.0 – 7.2 Add 10% $CaCl_2$ 3 ml/100 ml and 10% K_2HPO_4 - 2 ml/100 ml before pouring to the plates.)(11). The inoculated plates were incubated at 28-30ºC for 48 hrs. At the end of the incubation period number of colonies of *Bacillus megaterium* appearing on the plates were observed (11). The cultures so isolated were characterized through a number of morphological, microbiological and biochemical tests. Aerobic spore formers pasteurize a diluted soil sample at 80 degrees for 15 minutes, then plated onto nutrient agar and incubated at 37°C for 24 hrs. The plates were examined after 24 hrs. for typical colonies identified as catalase-positive, Gram-positive, endospore-forming rods (7).

***Bacillus megaterium* Growth and degradation Glph in MSM**

The hydrolysis capacity was measured (2,5,7,14,21,30,60)days by spectrophotometer OD $_{600}$, and the extraction of Glph residue from MSM were in 30 and 60 day by added equal volume from media and ethyl acetate as extraction reagent in tube with twice time extraction, The mixture was centrifuged at 3000 rpm for ten minutes. The ethyl acetate with residual Glph was filtered and dried with anhydrous sodium sulfate followed by filtration through glass-fiber paper (Whatman GF/B). This operation was conducted sequentially and the filtrates were mixed (10).The degradation ratio (%), were measured for Glph according to equation 1

$$\boldsymbol{P = \left(1 - \frac{C1}{C0}\right) x100\%} \qquad \textbf{(1)}$$

P= refered to the degradation rate of Glph ,

C1= account for Glph concentration of treated test sample.

C0= account for the control (13**).**

Metabolite analysis

Each of extraction by ethyl acetate were analyzed by HPLC .Chromatography determination were with a UV-Vis detector at 254 nm and a manual injector equipped with a 20-µL loop, using a C-18 ZORBAX column (5µm; 150 mm×4.6 mm i.d.) from Agilen Technologies as stationary phase. The mobile phase used was prepared by mixing acetic acid (1%) with methanol in a 60:40 ratio (v/v). The flow rate used was 1.0 mL min-1, stabilized at constant temperature 23–25°C (8).

RESULTS AND DISCUSSION

Morphological and Biochemical tests

Beside used the selective media, Sperber's Medium, The Morphological Test, Table 1 and biochemical tests, as in Table 2.

Table 1. The Morphological tests

Morphological tests	
Spore shape	**Rod-like/ flagella spores**
Colonies	**Round to irregular /yellow to brown or black after prolonged incubation**
Motility	**+**
Gram stain	**+**
Aerobic	**+**
Temperature	**3-20 °C/ 35-45°C,optimun30°C**
pH	**5.7- 7**

Table 2. The Biochemical tests

Biochemical tests			
Catalase	**+**	**Nitrate reduction / Degradation of tyrosine**	**+/-**
Starch Hydrolysis	**+**	**Casien hydrolysis**	**+**
Citrate utilization	**+**	**Indol/ Methyl Red**	**-**
Esculin hydrolysis	**+**	**Arginine dihydrolase**	**-**
Gelatin hydrolysis	**+**	**Tryptophan deaminase**	**-**
Oxidase	**+**	**Hydrolysis Urea**	**-**

***Bacillus megaterium* hydrolyzes and bacteria growth**

Growth of *B. megaterium*

The results show that the best growth of *B. megaterium* were in (60 days) for both (5 , 25) ppm (0.164, 0.167)respectively ,while the 15 ppm show the highest growth in 60 day (0.215) in comparative with others when used Glph as a carbon sources figure 1.

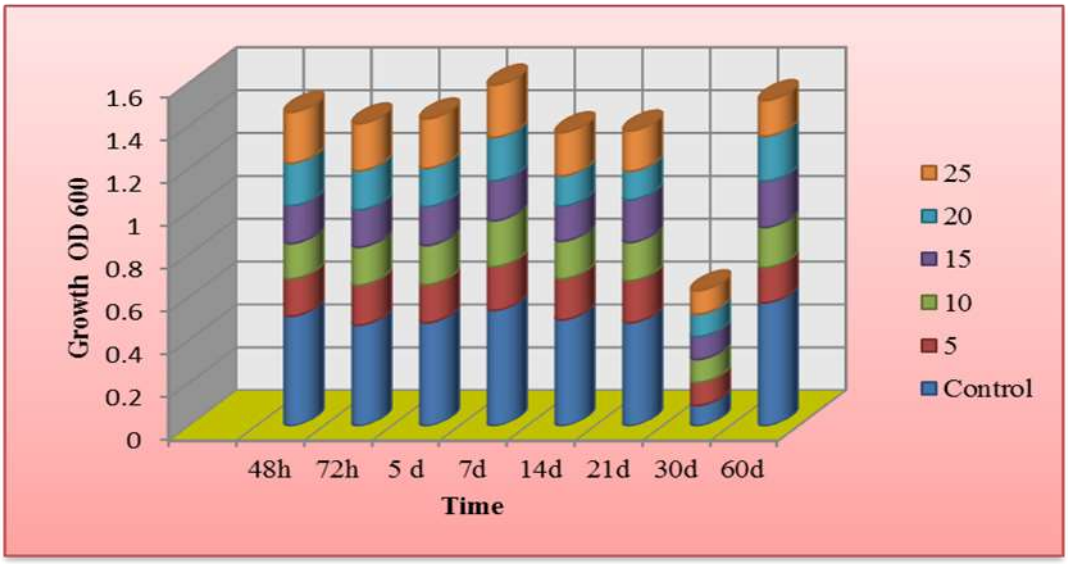
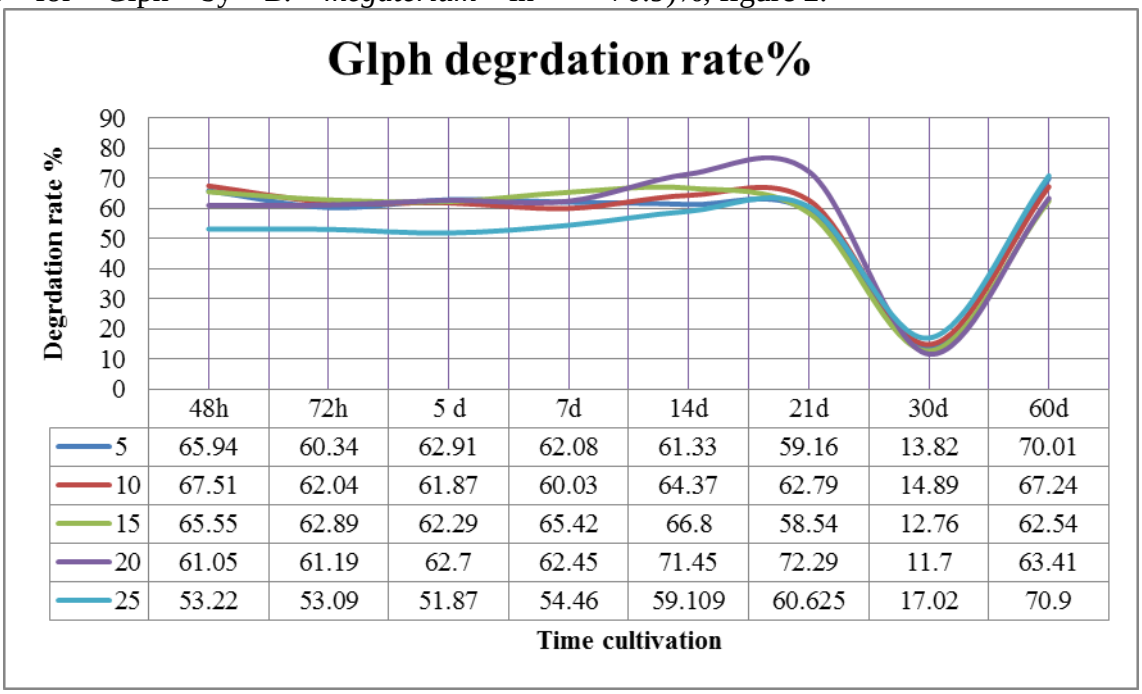


Figure 1. Growth of *B. megaterium* on MSM containing Glph

B. Degradation rate%

The results show that the best degradation rate% for Glph by *B. megaterium* in comparative among concentration were for both the 5-25 ppm in 60 day reached (70.01-70.9)%, figure 2.



	48h	72h	5 d	7d	14d	21d	30d	60d
5	65.94	60.34	62.91	62.08	61.33	59.16	13.82	70.01
10	67.51	62.04	61.87	60.03	64.37	62.79	14.89	67.24
15	65.55	62.89	62.29	65.42	66.8	58.54	12.76	62.54
20	61.05	61.19	62.7	62.45	71.45	72.29	11.7	63.41
25	53.22	53.09	51.87	54.46	59.109	60.625	17.02	70.9

Figure 2. Degradation rate of Glph in MSM in Comparative with control

Glyphosate residues by HPLC test

The study showed that B.M. has grown on (5,10,15,20,25)ppm concentration of Glph in MSM at 30 ℃ , as the growth of bacteria increased the concentration decreased generally in MSM with Glph in comparative with control in Fig (3,4).The best peak area that showed decreasing in Glph in 30 d were for concentration(5 ,10) ppm (7, 8)% ,while the 25 ppm showed 28% , while the results of Glph peak area for B.M. incubation for 60 days on MSM, showed the best for (20, 15) ppm in comparative with control. When compare among the Glph Concentration's via HPLC and degradation ratio% in Fig (5, 6), showed when increase the time incubation to 60 days , the *Bacillus megaterium* degradation ratio% increased for all Glph concentration's , but the best were for (5, 25)ppm for both the HPLC analysis and Degradation ration%.

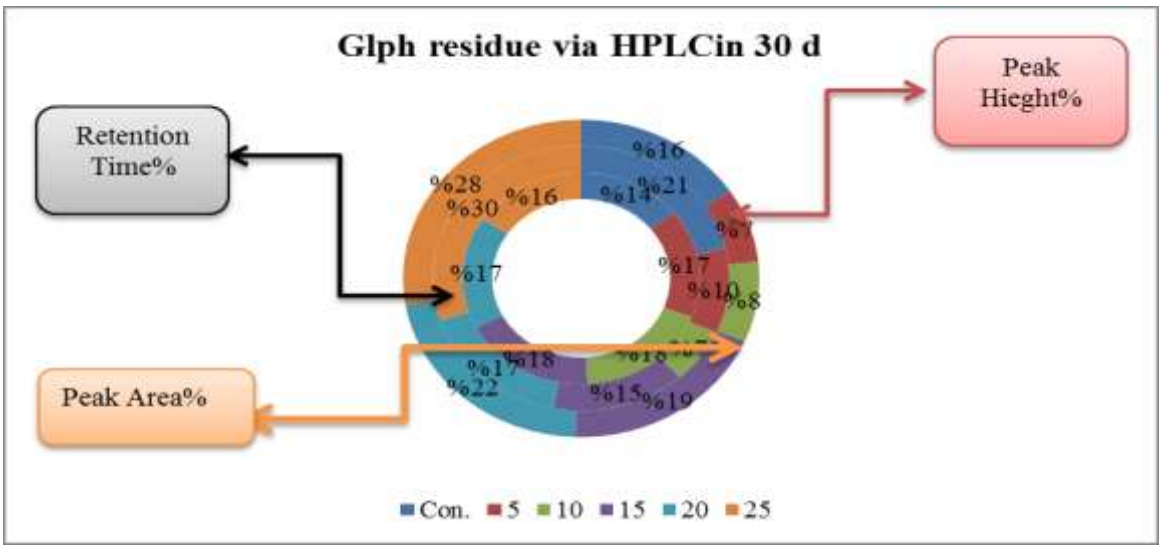


Figure 3. Retention time, peak area and peak height of (5,10,15,20,25 ppm+control) dilution of Glph after incubation B.M. 30days in MSM

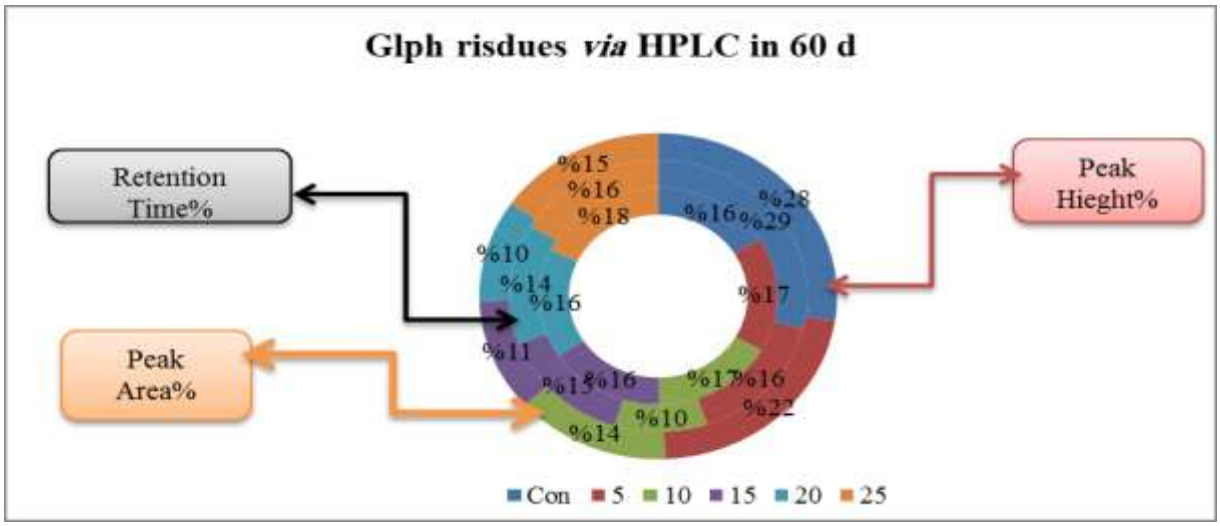


Figure 4. Retention time, peak area and peak height of (5,10,15,20,25 ppm+control) dilution of Glph after incubation B.M. 60days in MSM

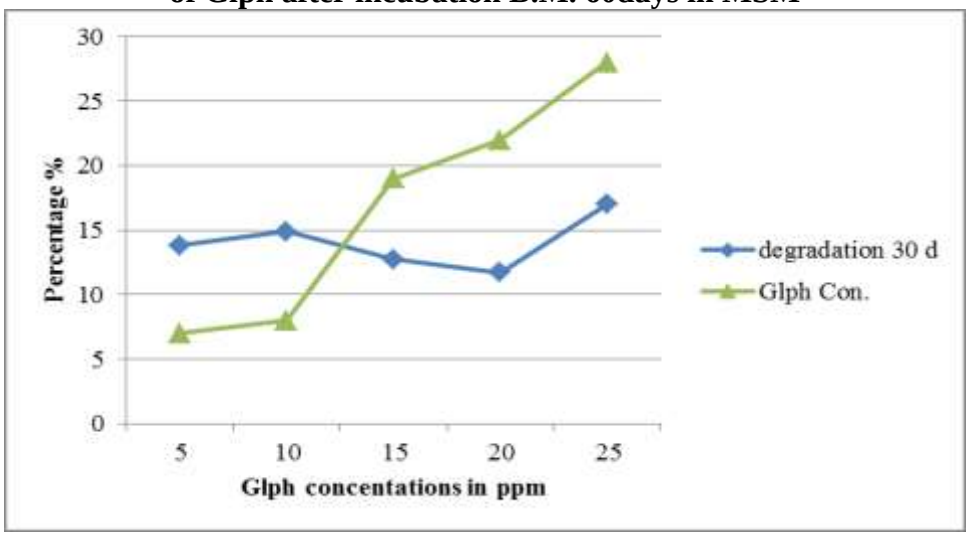


Figure 5. Comparative among the degradation ratio% and Glph concentrations via HPLC in 30 days incubation on MSM

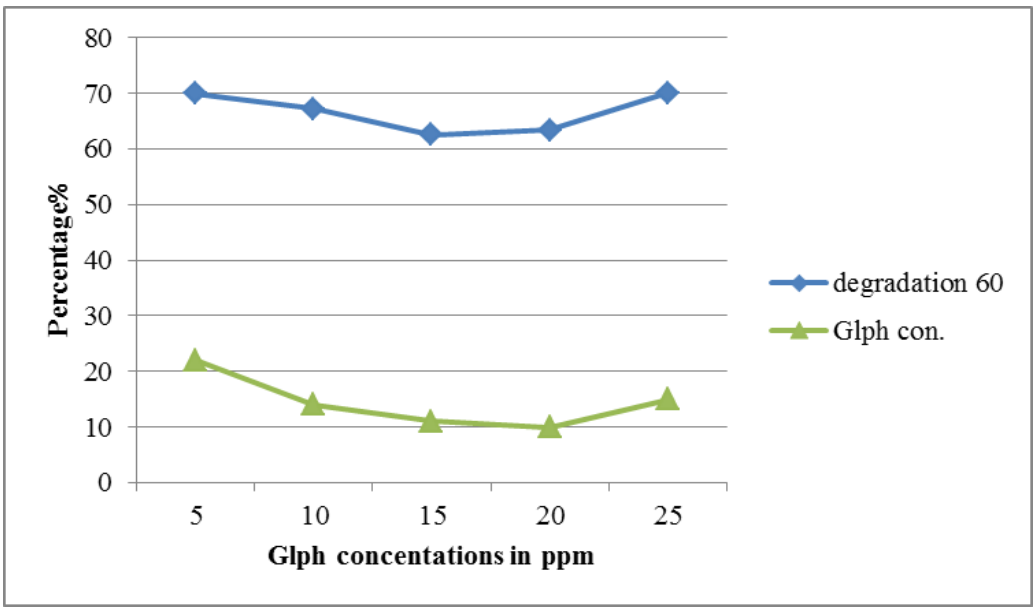


Figure 6. Comparative among the degradation rqtio% and Glph concentation via HPLC in 60 days incubation on MSM

Microbial degradation of organophosphorus pesticides and the development of bioremediation strategies for polluted agricultural soils based upon the introduction of biodegrading microorganisms, represent a growing area of research worldwide (15) . *B. megaterium* shows highest growth and degradation rate% in 60 days cultivation time for both 5,25 ppm concentration. The ability of degradation organophosphate pesticide like Chlorpyrifos by *B. megaterium* for 600 mgL-1 concentrations, was 81% in 10 days incubation (16), while B. M. show in 20 ppm /21days 72.29. In other study the B.M. Improve significant degradation ability towards atrazine (50 mg/kg) could reach 99.0% by the microbial agent after 7 days(17).The bacteria show increasing in growth with corresponding increase in glyphosate concentration while *B. subtillus* show reduction in growth with corresponding increase in glyphosate concentration(18). Other study, showed *B.megaterium* ability to degradation other organophosphore pesticides, Chlorpyrifos in 7 -14 days , will be potentially useful in abatement of Chlorpyrifos contaminated soil (19).Monocrotophos(MCP), also degraded to carbon dioxide, ammonium and phosphate through formation of unknown compound metabolic by *B. megaterium*, reached 83% (20). In this study, *Bacillus megaterium* was isolated from Iraqi Soils and identification by morphological and biochemical tests beside a Sperber's Medium as selectivity media to B.M. The best results for growth B.M. were in 48 h while in 60 days had the same growth for both 5,25 ppm on MSM. The degradation rate % ability were the best in (5,25) ppm /60 days (70.01-70.9)%.The Glyphosate Concentrations via HPLC and degradation ratio% , showed when increasing the time incubation to 60 days, the *Bacillus megaterium* degradation ratio% increased for all Glyphosate concentration's, but the best were for (5, 25)ppm for both the HPLC analysis and Degradation ration%. From all the conclusion is that the *B. megaterium* used the Glyphosate as source for carbon and phosphorus and suggest could be well exploited for bioremediation of Glyphosate contaminated sites.

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