Biodegradation of (N-phosphonomethyl)glycine Utilizing Bacillus subtilis using different incubation periods

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Abstract. The study aimed to evaluate Bacillus subtilis biodegradation one of organic-pesticides in different periods. Bacillus subtilis was isolated from Iraqi soils, identification by morphological and biochemical tests. The best growth of B. subtilis were in(72 hours) / (10-15) ppm (0.200-0.196)respectively ,while the 5 ppm showed the highest growth in 60 day (0.163) .The best degradation rate% were for 15 ppm/ 14 days (90.32)%. The evaluation of (N-phosphonomethyl)glycine residues concentration’s via HPLC and degradation ratio%, showed with increasing time incubation to 30 days , Bacillus subtilis degradation ratio% increased for (15)ppm , while the best 60 days / (25)ppm. From all the conclusion is that the B. subtilis used the Glyphosate as source for carbon and phosphorus and suggest could be well exploited for bioremediation of Glyphosate contaminated sites in 15ppm/30 days and 20 ppm/60 days.

1. Introduction

The (N-phosphonomethyl)glycine is chemical name of Glyphosate. It is one of an organophosphate and non-selective herbicide, applied to the leaves of plants for killing both broadleaf plants and grasses [1]. (N-phosphonomethyl) glycine 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 pest’s control, they can cause potential human and environmental in case of extensive us[2]. The genotoxicity and carcinogenicity studies for (N-phosphonomethyl)glycine was assessed. There was no convincing evidence for direct DNA damage in vitro or in vivo[1]. (N-phosphonomethyl)glycine 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 [2].

The ability of Microorganism to remove pollutants from contaminated sites is one of promising treatment method [3].As an alternative strategy , is supported because of their effectiveness, minimize hazardous , economic value and environmental safety is known bioremediation [4].Many researcher improved that different bacteria groups shown great ability of degradation Organophosphorus insecticides and others [5,6,7,8]. The microorganisms have strategies in degradation reaction towards pesticides in
soils that are co-metabolism, catabolism and metabolic enzymes [9]. To determine the fate for pesticides in environmental, the microbial degradation can be a base factor for. The study aimed to carried out and investigate the ability of local bacterial isolated to tolerate and degrade (N-phosphonomethyl)glycine in different concentrations and value the residue of it in extraction solution from media by HPLC.

2. Material and Methods

2.1 Chemical and reagents. Commercial pesticide“(N-phosphonomethyl)glycine/Glyphosate “was purchased from Iraqi market and other chemicals and reagents were in laboratories of Water and Environmental Directorate of Iraqi Ministry of Science and Technology-Baghdad branch. The Mineral Salt Media (MS M) used to examine (N-phosphonomethyl) -glycine degradation via B. subtilis (MSM): (0.2 g KH2PO4; 0.5 g K2HPO4 (sterilized separately at 125 °C for 25 min to prevent precipitation and later aseptically added to the rest of the salts); 1g(NH4)2SO4; 0.2 g MgSO4•7H2O; 0.2 g NaCl; 0.05 g CaCl2•2H2O; 0.025 g FeSO4•7H2O; 0.005 g Na2MoO4; 0.0005 g MnSO4 (pH 7.0 ± 0.3) [10]. Flasks (125 mL) were supplemented with Glph (Glyphosate) as the only carbon source. The Final Concentration of (N-phosphono-methyl)glycine(N-PMG) were (5, 10, 15, 20, 25 ppm) with 0.5 ml from inoculum bacteria in comparative with control.

2.2 Soil Samples collection. Samples were taken from the top 15 cm of soil, 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 [3].

2.3 Isolation and identification of Bacillus Subtilis from soils. Bacillus Subtilis was isolated by Nutrient agar 28 g /L. The inoculated plates were incubated at 28-30ºC for 48 hrs. At the end of the incubation period number of colonies of Bacillus subtilis appearing on the plates were observed[11]. The cultures so isolated were characterized through a number of morphological, microbiological and biochemical tests[3].

2.4 Bacillus subtilis Growth and degradation ratio%. The hydrolysis capacity was measured (2, 5, 7, 14, 21, 30, 60) days by spectrophotometer OD600. The extraction of N-PMG 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, centrifuged at 3000 rpm /10 min. The ethyl acetate with residual N-PNG was filtered, 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 N-PMG according to equation 1:

\[ P = \frac{(1-C_1/C_0) \times 100}{1} \]  

\[ P \] refered to the degradation rate of Glph.

\[ C_1 \] account for Glph concentration of treated test sample.

\[ C_0 \] account for the control [12]

2.5 HPLC detection(N-phosphonomethyl)glycine. 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 was prepared by mixing acetic acid (1%) with methanol in a 60:40 ratio (v/v). The flow rate was 1.0 mL min-1, stabilized at constant temperature 23–25°C[13].
3. Results

3.1 Morphological and Biochemical tests. The Nutrient agar (NB), used to study the morphological test, table (1) and biochemical tests as in table(2).

| Table 1. The Morphological tests |
|----------------------------------|
| Morphological tests              |
| Spore shape | Rod / flagella spores, has endospore and spherical |
| Colonies | Round to irregular /bright ,soft , whitish . |
| Motility | + |
| Gram stain | + |
| Aerobic | Obligate aerobic |
| Temperature | 25-35°C |
| pH | 8 |

| Table 2. the Biochemical tests |
|--------------------------------|
| Biochemical tests               |
| Catalase | + |
| Starch Hydrolysis | + |
| Citrate utilization | + |
| Esculin hydrolysis | + |
| Gelatin hydrolysis | + |
| Oxidase | Variable |
| Nitrate reduction | + |
| Fructos/Glucose/Glycerol | + |
| Tyrosine hydrolysis | - |
| Casien hydrolysis | + |
| Indol/ Methyl Red | - |
| Arginine dihydrolase | - |
| Adonitol | - |
| Hydrolysis Urea | - |
| Degradation of tyrosine | - |
| Arabitol | - |
| Lysine | - |
| Phenylalanine | - |

3.2 Bacillus subtilis capacity hydrolyzes N-PMG in MSM and bacteria growth.

3.2.1. Growth of B. subtilis consortium in MSM. The results showed, the best growth of B. subtilis were in(72hours) / (10-15) ppm (0.200-0.196)respectively ,while the 5 ppm showed the highest growth in 60 day (0.163) in comparative with others and control , when used N-PMG as a carbon sources, Figure (1).
3.2.2 Degradation rate %. The results show that the best degradation rate % for N-PMG by *B. subtilis* among concentrations were 15 ppm/14 days day reached (90.32) %.

3.3 (N-phosphonomethyl)glycine residues via HPLC. The study showed, *B. subtilis* has grown on (5, 10, 15, 20, 25) ppm concentration of N-PMG in MSM at 30 °C, as the growth of bacteria increased the concentration decreased generally in MSM with N-PMG in comparative with control, Fig (3,4). The biggest peak area that showed decreasing in 30 d/ (25) ppm (12)% while the 15 ppm showed 37% . In other hand, N-PMG peak area for *B. subtilis* incubation for 60 days on MSM, the best(10). The increasing incubation time to 30 days , the *Bacillus subtilis* degradation ratio% increased for N-PMG/ (15)ppm, while 60 days,(25)ppm the best HPLC analysis and Degradation ration%.
4. Discussion.

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[14]. *B. subtilis* shows highest growth and degradation rate% in 60/5,25 ppm. The ability of degradation organophosphate pesticide like Chlorpyrifos by *B. megaterium* for 600 mgL-1 concentrations, was 81% in 10 days incubation [15]. *Bacillus megaterium*. show in 20 ppm/21days 72.29. In other study the *B.megaterium*, improve significant degradation ability towards atrazine (50 mg/kg) could reach 99.0% by the microbial agent after 7 days[16]. The bacteria showed increasing in growth with corresponding increase in glyphosate concentration while *B. subtiliss* showed reduction in growth with corresponding increase in glyphosate concentration[17]. Other study, showed *B.megaterium* ability to degradation other organophosphate pesticides, Chlorpyrifos in 7-14 days, will be potentially usefull in abatement of Chlorpyrifos contaminated soil. [18].Monocrotophos(MCP), also degraded to carbon dioxide, ammonium and phosphate through formation of unknown compound metabolic by *B. megaterium*, reached 83% [19].
5. Conclusion.

In this study, Bacillus subtilis was isolated from Iraqi soils and identification by morphological and biochemical tests. The results showed that the best growth of B. subtilis were in (72 hours) for both (10-15) ppm (0.200-0.196) respectively, while the 5 ppm showed the highest growth in 60 day (0.163). When used N-PMG as a carbon sources. The best degradation rate% were 15 ppm/14 days (90.32)%. The N-PMG concentration’s via HPLC and degradation ratio% showed, The biggest peak area decreasing N-PMG 30 d/ 25 ppm (12)% , while the 15 ppm showed 37%. Bacillus subtilis degradation ratio% increased (15)ppm, but in 60 days, the best were for (25)ppm for both the HPLC analysis and Degradation ration%.

From all the conclusion is that the B. subtilis used the N-PMG as source for carbon and phosphorus and suggestion could be well exploited for bioremediation of N-PMG contaminated sites in 15ppm/30 days and 20 ppm/60 days.

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