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RESEARCH ARTICLE

Comparison between Some Bacteria and Fungi for Biodegradation of Glyphosate Herbicide in some Agriculture Soil in Iraq

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Abstract

Glyphosate is a compound used as herbicide in the control and killing of grasses and herbaceous plants. It can be used in agriculture to prepare fields before planting during crop development and after crop harvest. Because of its toxicity to non-target organisms, there is need to decontaminate glyphosate from contaminated soils and bioremediation is a very useful alternative to conventional cleanup methods. The success of this will depend on isolating bacteria and fungi with the ability to degrade glyphosate in a changing environment. The abilities of two bacterial species *Aeromonassobraia* and *Acinitobacter hemolyticus* and three fungal species *Aspergillu niger, penicilium notatum and Rhizopus sp.* to degrade glyphosate herbicide under varying environmental conditions were evaluated in this study. The isolates were screened for glyphosate utilization using mineral salt medium containing glyphosate as carbon source. Two bacterial isolates were tested for their ability to biodegradation of glyphosate in liquid medium identified by Vitek compact system. Three fungal isolates were tested for their ability to biodegradation of glyphosate in liquid medium CzapekDox and Potato Dextrose agar (PDA).The two bacterial isolates, *Aeromonassobraia* and *Acinitobacter hemolyticus* and three fungal isolates, *Penicillium notatum, Aspergillus niger, and Rhizopus sp.,* showed the capacity to utilize glyphosate efficiently and were therefore used for further biodegradation studies. The optimum conditions (incubation period, temperature) for biodegradation of glyphosate were examined. The obtained results indicated that the best incubation period was after 7 days for *Aeromonassobraia* while the best incubation period was after 9 days for *Acinitobacter hemolyticus* in mineral salt medium. Favorite temperature was 45^0C for both bacterial isolates. Microbial growth during the study was monitored by measuring the optical density at 660 nm. This study showed that *Aeromonassobraia and Acinitobacter hemolyticus* exhibited a high capacity to efficiently degrade glyphosate under the environmental conditions studied. The obtained results indicated that the best growth was *Aspergillus niger* after 15 days while the weak growth was *Penicillium notatum* after 15 days in Czapek Dox and Potato Dextrose agar (PDA). Fungal growth during the study was monitored by observed rang density growth. This study showed that *Aspergillusniger* best capacity to efficiently degrade glyphosate under the 50,100 mg/l. Other concentrations (200,300mg/l) inhibit growth for all fungal isolate. Thus, the organisms can be exploited for biodegradation of glyphosate.

Keywords: *Bacteria, Fungi, Biodegradation, Glyphosate, Herbicide.*

Introduction

The intensive use of herbicides in cropping systems is a general practice and thus a matter of environmental concern. This is as a result of the potential hazardous effects of these chemicals on soil biological processes, non-target organisms and pollution of streams and rivers through runoffs. The common herbicides in use include 2, 4-dicholorophenoxyacetic acid (2,4-D) (isoproplyamine salt of glyphosate) [1]. Glyphosate on its own may be relatively harmless to humans. It is however formulated with surfactants such as polyoxyethylene amine (POEA) which is more toxic than glyphosate alone [2].Glyphosate is a broad spectrum, non-selective herbicide used in the control or killing of grasses, herbaceous plants, including deep rooted perennial weeds, brush, some broad-leaf trees and some shrubs (United States Department of Agriculture [3]. Biodegradation by fungi is also known as mycodegradation.

Bioremediation in which fungi are employed is sometimes called mycoremediation, Fungi are ubiquitous in the environment, and the literature on fungal ecology is vast. Despite this, the reports of fungi in bioremediation are under-represented and as such represent the untapped potentials in fungal bioremediation Moreover, the biology and ecology of mycoremediation has rarely been examined [4].

Although most living organisms lack this metabolic route such that they would not be potentially affected by this herbicide, the environmental consequences of the widespread use of glyphosate have been reported. On application, glyphosate remains unchanged in the soil for varying lengths of time, as a result of its adsorption on clay particles and organic matter present in the soil [5]. The removal of glyphosate from the environment is usually by microbiological processes as chemical process of degradation is ineffective because of the presence of highly stable bonds (carbon-phosphorus bond) present in the compound. Bacteria degrade glyphosate via two general pathways leading to the intermediate production of either glycine oraminomethylphosphonate (AMPA) [6, 7].

Environmental Fate of Glyphosate

Soil

In general, glyphosate is moderately persistent in soil. The primary reason crops can be planted or seeded directly into treated areas following application is that glyphosate exhibits essentially no appear activity even when applied at high rates [2]. In the soil environment, glyphosate is resistant to chemical degradation, is stable to sunlight, is relatively non-leachable, and has a low tendency to runoff (except as adsorbed to colloidal matter). It is relatively immobile in most soil environments as a result of its strong adsorption to soil particles. Gimsing *et al.,* 2004 found that less than one percent of the glyphosate in the soil is absorbed via the roots.

The primary metabolite of glyphosate is aminomethylphosphonic acid (AMPA). Degradation of AMPA is generally slower than that of glyphosate possibly because AMPA may adsorb onto soil particles more strongly than glyphosate and/or because it may be less likely to permeate the cell walls or membranes of soil microorganisms [3, 8]. The main objectives of the present study are the isolation and identification of glyphosate utilizing bacteria and fungi from agriculture soil using an enrichment culture technique, assessment of the growth response of the isolates in liquid medium containing glyphosate as carbon source on the isolates.

Material and Method

Chemicals Used

The glyphosate known as herbicide (containing360 g active ingredient/L of glyphosate) was purchased from a local market in Baghdad/Iraq. All other chemicals were of the highest purity commercially available.

Collection of Soil Samples

Soil samples were obtained from agriculture soil located at mony stat in Baghdad. These agriculture soils are known to have been previously exposed to glyphosate-based formulation (trade) for long periods of time. Soil samples were collected from depths of 0 - 15 cm from three different sites in each of the locations. Soil samples from each site were thoroughly mixed. All samples were placed in sterile polyethylene bags and taken immediately to the laboratory and stored at 4°C before use within 24 hr [9, 10].

Isolation Medium

A modified mineral salts medium (MSM) of consisting of (g/l) $(NH4)_2SO4$, 0.375; MgSO4, 0.075; $CaC0_3$, 0.03; FeSO4.7H2O, 0.001; MnSO4, 0.000001 and buffer, pH was adjusted to 7.0. All glass wares were washed with deionized water to remove contaminating before use. The medium was autoclaved at 121°C and 15 psi for 15 min prior to the addition of the filter sterilized (isopropylamine salt of glyphosate) as carbon source [11, 12, 13].

Isolation and purification of fungal strain from soil samples using both Czapek Dox and Potato Dextrose agar (PDA) media was carried out according to the method described by. Pure colonies were picked, streaked on slants of Potato Dextrose Agar, PDA kept at 4°C until using. For sub culturing, spores from the slants were suspended in sterile saline containing 0.01% Tween 80, gently agitating the tube with a Tween 80 solution to give a count within rang $3.0x10^6$ -$3.0x10^7$ spores/ml.

Spores suspension used for the inoculation was prepared by subcultures on the potato dextrose agar (PDA) and incubated at 28-30ºC for 7-10 days [4, 14].

Isolation of Glyphosate Utilizing Bacteria

The soil samples were air-dried and sieved using a 2 mm mesh. 5 g of each soil sample was suspended in 250-ml Erlenmeyer flasks containing a mixture of 50 ml of mineral salts medium and 1 ml of 10 mg/ml of glyphosate. This concentration was used because it is equivalent to the field application rate. The flasks were incubated on a rotary shaker at 120rpm for 5 days at 30°C. The above steps were repeated by taking 1.0 ml of sample from each broth culture and transferred to fresh enrichment medium followed by incubation as described for 5 days.

Isolation was done using the spread plate method on the solid mineral salts medium described above with added glyphosate. The plates were incubated at 30°C for 5 days. Morphologically distinct colonies were re-isolated and were repeatedly sub-cultured on nutrient agar. Identity of the isolates was affirmed after characterization by standard bacteriological methods [15, 16] and confirm by Vitec system. Stock cultures were maintained in nutrient agar slants at 4°C.

Inoculums Preparation and Standardization

Inoculate used for the study were prepared by inoculating isolates into nutrient broth and incubated at 30°C for 24 hr. using sterile normal saline the cells from the above cultures were resuspended to optical density of 0.5 at 660 nm).

Determination of Temperature Course of the Growth of *Aeromonassobraia and Acinitobacter hemolyticus*

1.0 ml portion of each isolate was inoculated into 150 ml of the screening medium (contained in a 500-ml flask) which is the isolation medium. It contained 3 ml of (10 mg/ml of glyphosate). The flasks were incubated 25,35,40,45. The ability of each isolate to utilize glyphosate was measured based on the turbidity of the medium at 660 nm using a spectrophotometer.

Determination of time Course of the Growth of *Aeromonassobraia and Acinitobacter Hemolyticus*

500-ml Erlenmeyer flasks containing 150 ml of the sterile screening medium was prepared and 3 ml 10 mg/ml of glyphosate was added to each flask. 1 ml of the inoculum (0.5) of each selected isolate was used to inoculate each flask (experiments were carried out in 3 replicates). The two isolates used were selected based on their utilization and tested incubation period. The medium was then incubated at 30°C for 192 hr. on a shaker at 120 rpm. 5 ml of the culture medium was collected from each flask at 12 h intervals and assayed for growth by measuring the optical density at 660 nm using a spectrometer.

Identification and Characterization of Fungal Isolates

The most efficient herbicides degrading fungal genera were identified in the Laboratory of Fungal and Bacterial Plant Diseases Researches, Plant Protection Department, College of Food and Agriculture Sciences, University of Bagdad, Iraq.

Screening of Filamentous Fungal Isolates and Growth Capability on Glyphosate

Fungal screening was carried out by monitoring the growth capability on Glyphosate different concentrations (0, 50, 100, 200 and 300 ppm) in Czapek Dox broth medium ($NaNO_3$3.0 g, K_2HPO_4 1.0g, KCl0.5g, $MgSO_4$ 0.1g, $FeSO_4.2H_2O$ 0.1g, Sucrose 30g, pH 6.5) for 15 days [17, 18].

Mycoremediation Experiments

To determine the efficiency of selected fungal strains for bioremediation of the herbicide, Czapek Dox broth medium containing only 1% sucrose and supplemented with different concentrations (from 50 to 300 ppm) of herbicide (Glyphosate) was prepared. The medium pH was adjusted at pH 6.5 and sterilized. The sterilized media were inoculated with selected fungal strains ($3.0x10^6$ - $3.0x10^7$ spore/ml), then incubated in rotary shaker operating at 150 rpm at 30°C for16 days [19,20].

Result and discussion

Effects of Temperature on the Growth of *Aeromonassobraia and Acinitobacter Hemolyticus*

Regarding temperature, both bacterial isolates examined at various temperature values with glyphosate biodegradation are shown in Table1.

Apparently, highest growth values in the both bacterial isolates under various temperature values were recorded in case of glyphosate herbicide in 45C ^{0} while much lower growth values were found with 35C ^{0}.Table 1

Table 1: Effects of temperature on the growth of *Aeromonassobraia*

Aeromonassobraia	
Temp. (C)	O.D (660nm)
25	1.035
35	0.06
40	0.36
45	1.82

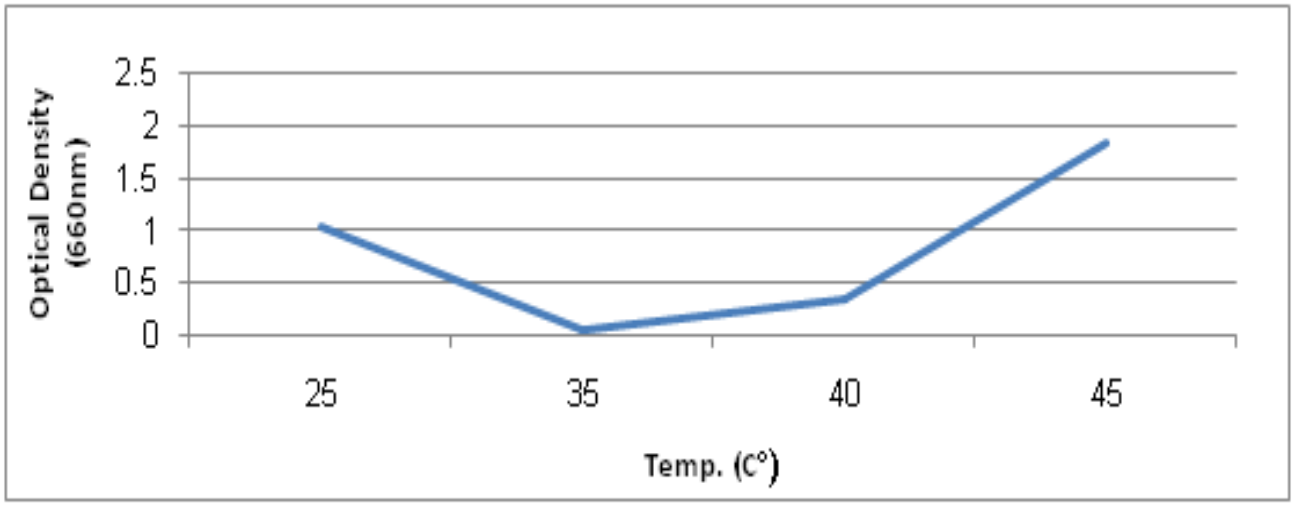

Figure1: Effect temperature and Optical Density (660nm) for *Aeromonassobraia*

The stimulating effect of temperature on degradation of glyphosate by *Aeromonas sobraia* was verified and consistent with previous studies [21, 22].

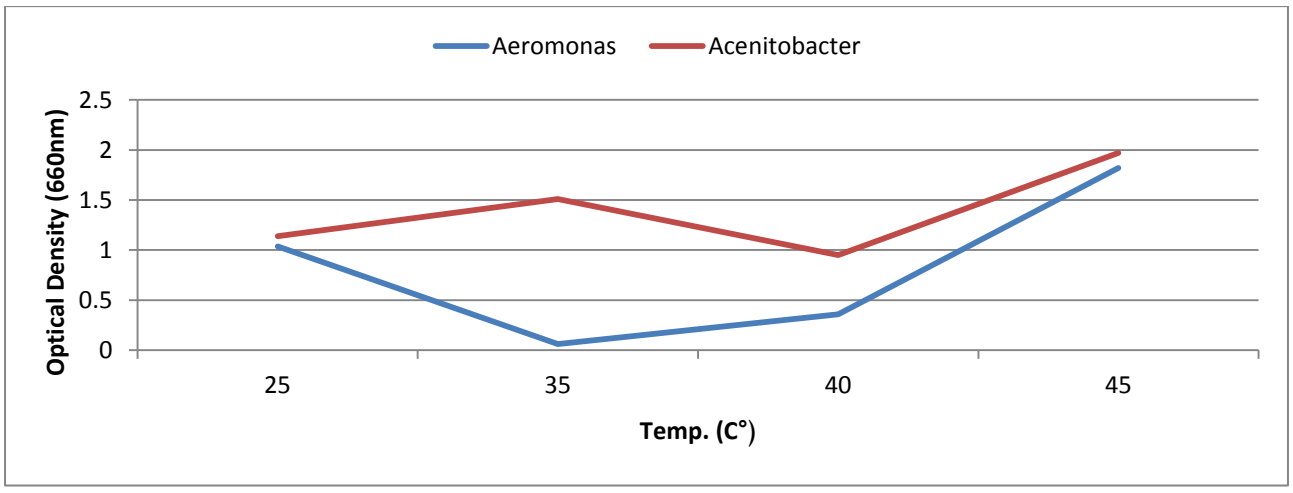

Figure2: Effect Temperature and Optical Density (660nm) for *Aeromonassobraia*and*Acinitobacterhemolyticus*

Effects of Time on the Growth of *Aeromonassobraia*

Different bacterial isolates that incubated at various incubation periods with glyphosate herbicide are given in Table 2. Result show this bacteria give good growth after 7 days.

Table 2: Effects of temperature on the growth of *Aeromonassobraia*

Aeromonas	
Time (days)	O.D (660nm)
1	0.5
5	1.49
7	1.87
9	1.67
10	1.59

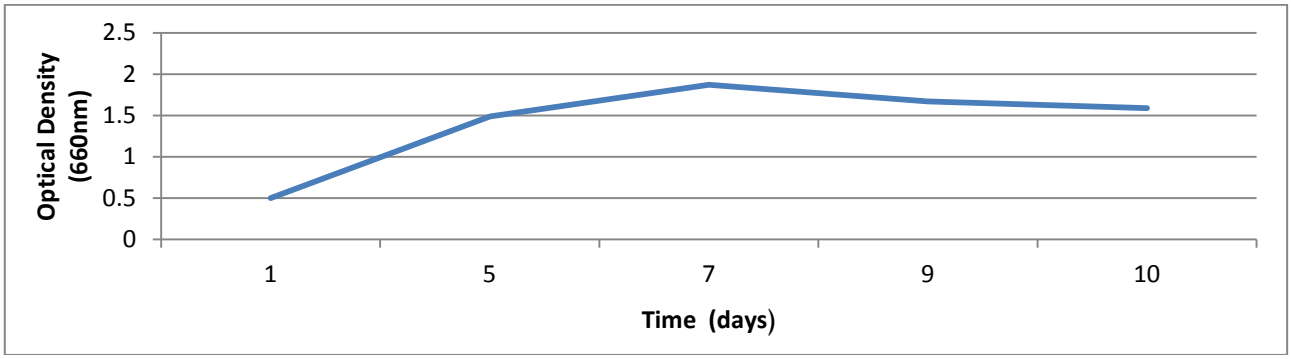

Figure 3: Effect Time and Optical Density (660nm)*Aeromonassobraia*

Effects of Time on the Growth of *Acinitobacterhemolyticus*

Different bacterial isolates that incubated at various incubation periods with glyphosate herbicide are given in Table 3. Result show this bacteria give good growth after 9 days.

Table 3: Effects of temperature on the growth of *Acinitobacterhemolyticus*

Acenitobacter	
Time (days)	O.D (660nm)
1	0.5
5	1.39
7	1.44
9	1.54
10	1.48

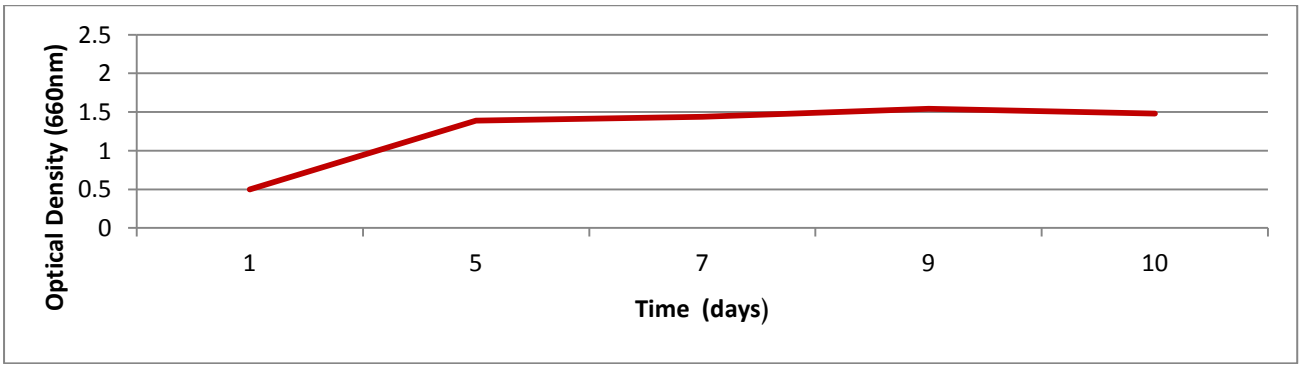

Figure 4: Effect Time and Optical Density (660nm)*Acinitobacterhemolyticus*

Glyphosate is microbial degraded in soil and water and has a reported field half-life of 47 days and a laboratory half-life of < 25 days. Studies of glyphosate degrading bacteria have involved selection for, and isolation of pure bacterial strains with enhanced or novel detoxification capabilities for potential uses in biotechnology industry and biodegradation of polluted soils and water [23, 24].

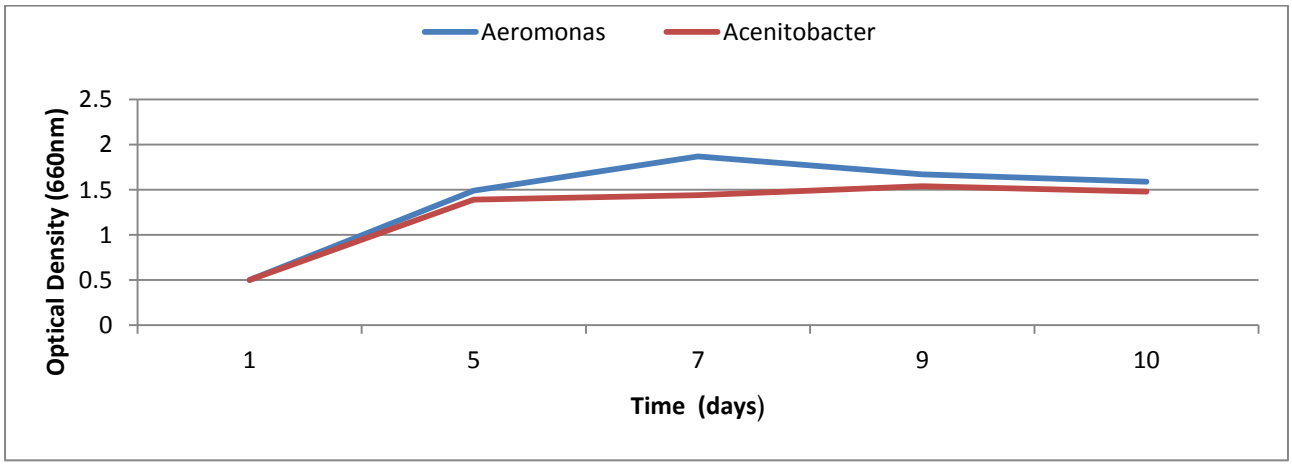

Figure5: Effect time and Optical Density (660nm) for *Aeromonassobraia*and*Acinitobacterhemolyticus*

The herbicide is inactivated and biodegraded by soil microbes at rates of degradation related to microbial activity in the soil and factors that affect this. The biological degradation process is carried out under both aerobic and anaerobic conditions by soil micro flora. Rates of decomposition depend on soil and micro floral population types [25].
The results suggested that the bacterial isolates were not forming any toxic intermediates during the degradation of pesticide and thus could be utilized in the bioremediation process of pesticide contaminated soil [26].

Effects Concentration on the Growth of *Aspergillu Niger, Penicilium Notatum and Rhizopus*

Regarding [0, 50, 100, 200, 300] mg/L concentration of glyphosate with *Rhizopus sp*. After incubation period that appear all containers is growing in few numbers Figure 6. Forty-five fungal isolates to grow in the presence of chosen herbicide at used concentrations are varied. In addition, the fungal growth was inversely proportional with the herbicide concentration [17].

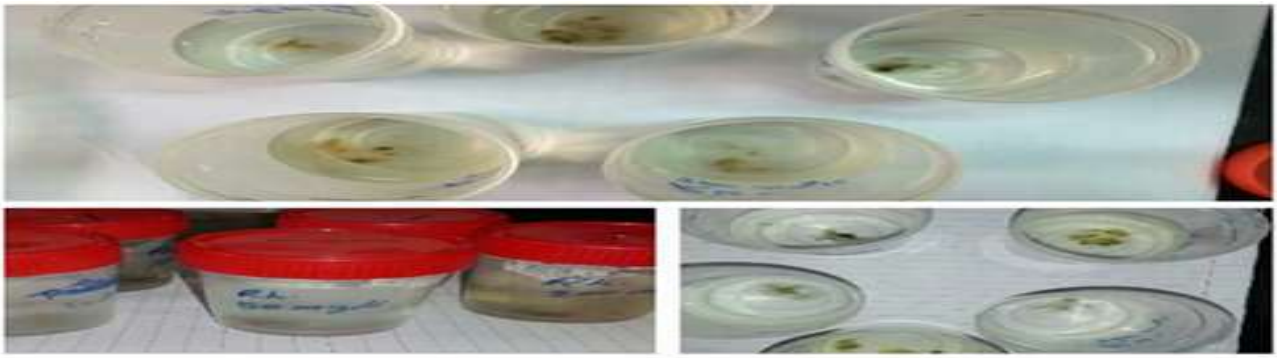

Figure 6: Biodegradation of Glyphosate by *Rhizopusspp*

Biodegradation of Glyphosate by *Penicilliumnotatum*, the result shows ability growth in 50,100,200 mg/l and no growth in 300 mg/l after incubation period Figure 7.

Figure 7: Biodegradation of Glyphosate by *Penicilliumnotatum*.

Figure 3 shows containers of [0, 50, 100, 200, 300] mg/L concentration of glyphosate as control after incubation period.

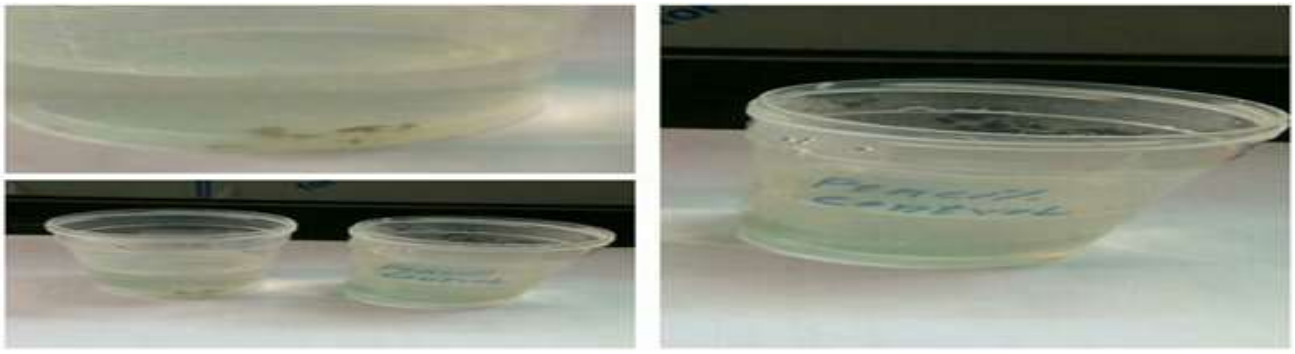

Figure 8: Biodegradation of Glyphosate by *Penicilliumnotatum*.

Aspergillusniger appear in 100 mg/l growing compare with 200, 300 mg/L of glyphosate after incubation period Figure 9.

Figure 9: Biodegradation of Glyphosate by *Aspergillusniger*

Biodegradation of Glyphosate by *Aspergillusniger* appears weak growth 50 mg/L after incubation period Figure 10.

Figure 10: Biodegradation of Glyphosate by *Aspergillusniger*

Aspergilligroup are commonly found in soil and on decaying organic material. By converting resistant organic chemicals such as pesticides into simplified metabolites and eventually into soluble benefits molecules, fungi such as Aspergillus spp. Play an important role in carbon cycling [17]. The overall aim is to examine the manner and function of fungal pesticide degradation. First, biological characteristics of the fungi which are relevant for successful bioremediation will be highlighted [19]. Filamentous fungi studies into the bioremediation potential of microorganisms have for the largest part focused on bacterial degraders. However, filamentous fungi possess characteristics which are advantageous in heterogeneous environments. Although fungi are non-motile they can respond quickly to changing environmental conditions to survive or escape them [19]. Abiotic factors that primarily affect mycelial growth includes temperature, water potential, pH, oxygen accessibility, and nutrient status, however, filamentous fungi can escape unfavorable conditions which in heterogeneous environments give them an advantage [4].

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