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The Utilization of Glyphosate by Bacteria Isolated from Soil

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Abstract: Glyphosate is one of the most commonly used herbicides worldwide. It is primarily applied to agricultural lands. This study examined the utilization of glyphosate by bacteria isolated from soil. Five bacteria were isolated, namely; Bacillus cereus, Bacillus subtilis, Escherichia coli, Pseudomonas sp., and Actinomyces sp. Bacillus cereus and Bacillus subtilis were selected for the studies based on their rapid degradation of the herbicides. The ability of the isolates to degrade different concentrations of glyphosate were tested in minimal salt medium (MSM) and incubated on a rotary shaker at 120 rpm at 30ºC for 28 days. The effects of Pb^{2+} and Cd^{2+} on degradation of the isolates were also determined at concentrations of 200 µg/ml, 300 µg/ml and 400 µg/ml in 150 ml of the MSM. The bacteria were isolated using pour plate method and identified based on their cultural and biochemical characteristics. The two isolates were identified as Bacillus cereus BFM4 and Bacillus subtilis H184 using polymerase chain reaction and sequence analysis. There were significant differences ($P < 0.05$) in the percentage utilization of the herbicides by the test organisms in all the treatments at day 28. Bacillus cereus BFM4 had the highest percentage utilization of 97.04 % and 90.49 % of glyphosate at the lowest concentration 20 mg/ml and 400 µg/ml of Pb^{2+}. The results of this study showed that the isolates were able to utilize varying concentration of glyphosate with an increased utilization on addition of Pb^{2+} and Cd^{2+}.

Keywords: Utilization, Bacillus cereus, Bacillus subtilis, glyphosate, heavy metals.

Introduction

Herbicides are used extensively in agriculture for the control of many annual and perennial weeds [1]. However, the indiscriminate use of herbicides may result in weed resistance or alter the biological functions of soil; additionally,

herbicides can have extensive unintended effects on nutrient availability and disease severity [2] resulting from direct herbicide-induced weakening of plant defenses and increased pathogen population and virulence

Herbicides applied to the environment have shown to have long term residual effects while others have shown to have acute fatal effects when not properly handled. Organochlorine herbicides for example have shown to be persistent in the environment, the result of which find their way to contaminate ground water, surface water, food products, air, soil [3]. Herbicides can enter the human body through inhalation of aerosols, dust and vapor that contain herbicides; through oral exposure by consuming contaminated food and water; and through dermal exposure by direct contact of pesticides with skin [1].

The effects of herbicides on human health are harmful based on the toxicity of the chemical and the length and magnitude of exposure [4]. Farm workers and their families experience the greatest exposure to agricultural pesticides through direct contact with the chemicals. Children are most susceptible and sensitive to herbicides due to their small size and underdevelopment. The chemicals can bioaccumulate in the body over time. Exposure to pesticides can range from mild skin irritation to birth defects, tumors, genetic changes, blood and nerve disorders, respiratory diseases, reproduction disorders, endocrine disruption, and even coma or death [5]. It is this aspect of herbicide in the environment that has raised concern among environmental scientists to study their behaviour in the environment and then come out with a sound alternative so as to rescue the human population from their adverse effects.

Herbicide may eliminate plants and animals essential to the functioning of the entire, promote the dominance of undesired species, or may simply decrease the number and variety of species present in the community. This may disrupt the dynamics of the food webs in the community by breaking the existing dietary linkages between species. This has created a further dependence on herbicides. The effects of herbicides on the biodiversity of plants and animals in agricultural landscapes, whether caused directly or indirectly by herbicides, constitute a major adverse environmental impact of herbicides [1].

Glyphosate (N-(phosphonomethyl) glycine), which is commonly called Roundup is one of the most commonly used herbicides worldwide [6]. It is primarily applied to agricultural lands. Glyphosate is also popular in production forestry because of its effectiveness in controlling many understory plant species, benign effects on conifers, low mammalian toxicity, and rapid inactivation in soil [1]. It is a phosphorus containing amino acid that functions both as a sole phosphorus source for in vitro microbial growth and as a readily available carbon and nitrogen source when degraded in soil.

The potential non-target effects of glyphosate on soil microorganisms and their processes, such as nutrient cycling and maintenance of soil structure, are

of concern. Glyphosate inhibits protein synthesis via the shikimic acid pathway in bacteria and fungi and one of its surfactants, polyoxyethylene tallow amine, is toxic to species of bacteria and protozoa [7].Because of the impact of herbicides on soil microbial ecosystems and agriculture, it is important to identify methods for enhancing herbicide degradation. Several treatment processes are available for removing herbicides, including biodegradation, filtration, adsorption, membrane technique, chemical, hydrolytic, and photolytic degradation [8].

Biodegradation of herbicides by microbial agents such as bacteria is an eco-friendly, cost effective, highly efficient approach and can be considered as a superior alternative to physical and chemical methods which are not only technically laborious and costly; also are not sufficient to completely degrade organic toxins present in herbicides. Microorganisms have a lot of metabolic diversity which makes it easy for them to metabolize herbicides. Microorganisms has the capacity to completely mineralize herbicides into simpler compounds such as carbon dioxide (CO_2) and water (H_2O) which are considered as non-harmful byproducts. Biodegradation of herbicides is important in terms of sustainability, because the microbial agents that are involved in carrying out the biodegradation process are always in the environment and so one does not need to buy them from the market [8, 9]. The use of bacteria (e.g. species of Pseudomonas, Bacillus) for degradation and detoxification of numerous toxic chemicals such as herbicides is an effective tool to decontaminate the polluted agricultural sites. Isolation of indigenous bacteria and yeasts capable of metabolizing herbicides provides environmentally friendly means of in situ detoxification. Many herbicide-degrading genes in soil bacteria have been reported to be encoded on plasmids [5]. The aim of this research therefore was to study the growth of some soil microorganisms in glyphosate enriched minimal salt medium.

Materials and Methods

Collection of Samples

A soil sample was collected with a sterile soil auger from a depth of 0-15 cm from four different sites within Federal University of Technology Botanical Garden, Bosso campus, Minna. Soil samples from each site was thoroughly mixed and placed in sterile polyethylene bags. This was transported immediately to Microbiology laboratory of the University and stored at 4^{o}C before use within 72 hours [10]. Glyphosate 41% SL was purchased from Brains and Strength Company limited, Maikunkele, Minna, Nigeria. The choice for this herbicide was because it is commonly used for the control of annual and biennial weeds in the study area.

Preparation of Isolation Medium

A Mineral Salts Medium (MSM)consisting of $MgSO_4.7\ H_2O$, 0.2g; K_2HPO_4, 1.8g; $FeSO_4.7H_2O$, 0.01 g;NH_4Cl, 4.0 g; NaCl, 0.1 g; KH_2PO_4, 1.2 g in 1000 ml of distilled water was used. All glass wares were washed with 1M HCl and thoroughly rinsed with deionized water to remove contaminating phosphate before use.

The medium was autoclaved at 121ºC for 15 minutes [11].

Isolation of Herbicides Utilizing Bacteria

Herbicide utilizing bacteria were isolated by air-drying soil samples and sieving it using a 2 mm mesh. A soil sample (5 g) was suspended in 250 ml Erlenmeyer flask containing a mixture of 50 ml of mineral salts medium and 1 ml of the herbicide. The flask was incubated on a rotary shaker at 120 rpm for 24 hours at 30ºC. Isolation of bacteria was done using the pour plate method. The plates were incubated at 37^0C for 24 hours. Morphologically distinct colonies were isolated and repeatedly sub-cultured on nutrient agar. Identity of the isolates was affirmed after characterization by standard bacteriological methods and molecular technique.

Identification and Characterization of Bacterial Isolates

The isolates from the screening procedure were identified using microbiological and biochemical procedures such as Gram staining, spore staining, motility and citrate utilization tests [12].

Molecular Characterization of Bacteria Isolate

The molecular method employed included DNA extraction, amplification of copies of DNA using polymerase chain reaction using 16S rRNA gene. The 16S rRNA gene sequences were used in BLAST searches to determine the best similarity to sequences in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Screening of Isolates for Herbicides Utilization

The isolates for herbicide utilization were screened by inoculating 1.0 ml portion of each isolate into 150-ml of mineral salt medium (contained in a 250-ml flask). It contained different concentrations of the herbicide (20, 30, 40 mg/ml of the herbicide). The flasks were incubated on a rotary shaker at 120 rpm for 180 h at 30ºC [13]. The ability of each isolate to utilize the herbicide was determined by spectrophotometry (Shimadzu UV, Model: UV-180, Japan) at 482 nm.

Effects of Different Concentrations of Heavy Metals on the Bacterial Isolates

Salts of the selected heavy metals ions Pb^{2+} and Cd^{2+}, at a concentration of 200 µg/ml, 300 µg/ml and 400 µg/ml were added to 150 ml of the mineral salts medium. The medium was autoclaved prior to the addition of different concentrations (20, 30, 40 mg/ml) of the filter-sterilized herbicides (size of the filter is 0.045 mm in diameter). Aliquot (1 ml) of each isolate was inoculated into the media and incubated at 30^0C for 28 days. Medium without heavy metals was used as control. Inoculated flasks were incubated at 30^0C on a rotary shaker at 120 rpm. Growth was assayed by measuring the optical density with a spectrophotometer (Shimadzu UV, Model: UV-180, Japan) at 482 nm [4].

Statistical Analysis

Statistical analysis of the data generated was carried out to indicate mean significant differences between the treatments using Statistical Package for Social Sciences (SPSS) version 16.0. One way analysis of variance (ANOVA) was used. Graphs and tables were used for data presentation.

Results

Bacterial Isolates Screened for Glyphosate Utilization

Five bacterial isolates were screened for glyphosate utilization by measuring their growth at 482 nm on medium containing glyphosate as sole carbon and phosphorous source. Of the five identified bacterial species, two (Bacillus subtilis and Bacillus cereus) were selected for further biodegradation studies based on their rapid utilization of glyphosate (Figure 1).

Molecular characteristics of bacterial isolates

The molecular characteristics of the bacterial isolates with the best ability to degrade the herbicide was determined as observed in Figure 2 showing the integrity of the amplified genes on agarose gel. The gel documented images of the isolated bacterial DNA after electrophoresis appeared at 1500 kb which indicated pure isolates. Lane Mk represents molecular marker (ladder). The result of the sequence analysis confirmed the isolates as Bacillus cereus strain BFM4 and Bacillus subtilis strain H184 using sequence analysis.

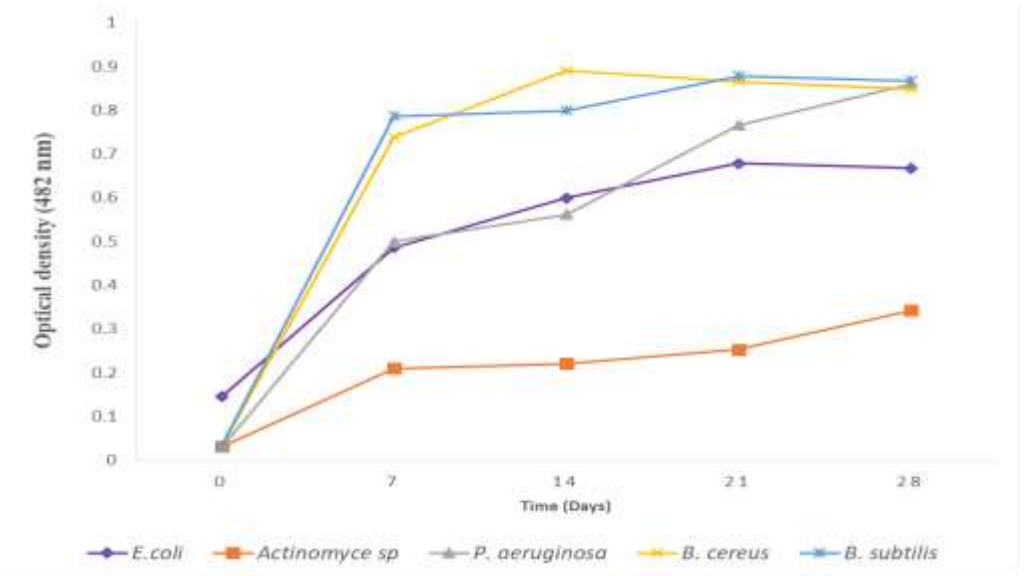

Figure 1: Bacterial isolates screened for glyphosate utilization

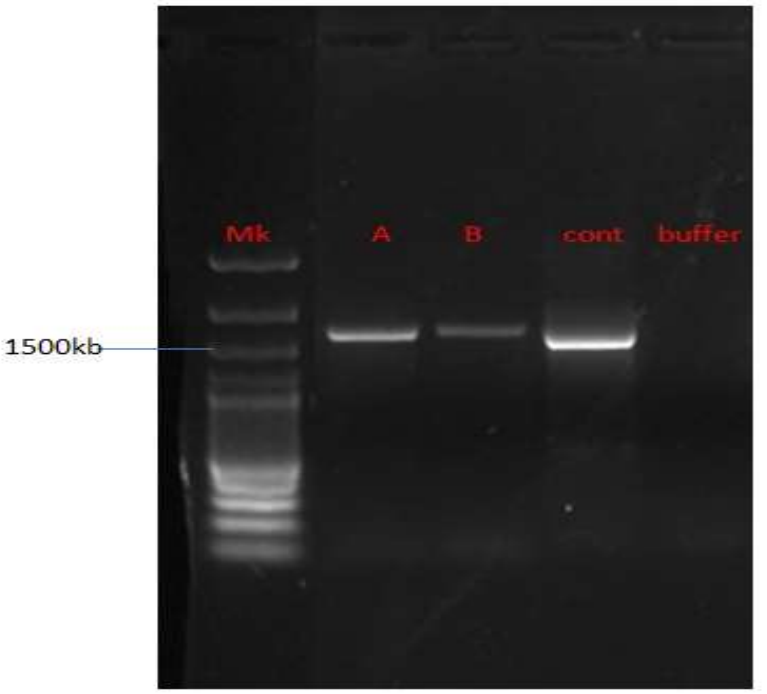

Figure 2: Capture gel electrophoresis showing size of the amplified genes
Keys:
A= *Bacillus cereus* strain BFM4, B= *Bacillus subtilis* strain H184, Control= *Lactobacillus plantarum*, Buffer= tris base, acetic acid, EDTA

Nucleotide sequence ofBacillus cereus strain BFM4 is as follows:

GGGCGCACTATACATGCAAGTCG
AGGGGACAGATGGGAGCTTGCTT
CCTGATGTTAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTGCC
TGTAAGACTGGGATAACTCCGGG
AAACCGGGGCTAATACCGGATGC
TTGTTTGAACCGCATGGTTCAAAC
ATAAAAGGTGGCTTCGGCTACCA
CTTACAGATGGACCCGCGGCGCA
TTAGCTAGTTGGTGAGGTAATGG
CTCACCAAGGCAACGATGCGTAG
CCGACCTGAGAGGGTGATCGGCC
ACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGT
AGGGAATCTTCCGCAATGGACGA
AGTCTGACGGAGCAACGCCGCGT
GAGTGATGAAGGTTTTCGGATCG
TAAAGCTCTGTTGTTAGGGAAGA
ACAAGTACCGTTCGAATAGGGCG
GTACCTTGACGGTACCTAACCAG
AAAGCCACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGT
GGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGGGCTCGCAGGCGG
TTTTTAAGTCTGATGTGAAAGCCC
CCGGCTCAACCGGGGAGGGTCAT
TGGAAACTGGGGAACTTGAGTGC
AGAAGAGGAGAGTGGAATTCCAC
GTGTAGCGGTGAAATGCGTAGAG
ATGTGGAGGAACACCAGTGGCGA
AGGCGACTCTCTGGTCTGTAACTG
ACGCTGAGGAGCGAAAGCGTGGG

GAGCGAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATG
AGTGCTAAGTGTTAGGGGGTTTCC
GCCCCTTAGTGCTGCAGCTAACGC
ATTAAGCACTCCGCCTGGGGAGT
ACGGTCGCAAGACTGAAACTCAA
AGGAATTGACGGGGGCCCGCACA
AGCGGTGGAGCATGTGGTTTAAT
TCGAAGCAACGCGAAGAACCTTA
CCAGGTCTGACATCCTCTGACAAT
CCTAGAGATAGGACGTCCCCTTC
GGGGGCAGAGTGACAGGTGGTGC
ATGGTTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGATCTTAGT
TGCCAGCATTCAGTTGGCACTCTA
AGGTGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGATGACGTCAA
ATCATCATGCCCCTTATGACCTGG
GCTACACACGTGCTACAATGGAC
AGAACAAAGGGCAGCGAAACCG
GAGGTTAAGCCAATCCCACAAAT
CTGTTCTCAGTTCGGATCGCAGTC
TGCAACTCGACTGCGTGAAGCTG
GAATCGCTAGTAATCGCGGATCA
GCATGCCGCGGTGAATACGTTCC
CGGGCCTTGTACCCAACCGCCCGT
CACACCACGAGAGTTTGTAACAC
CCGAAGTCGGTGAGGTAACCTTTT
AGGAGCCAGCGGCCGAAGGTGGC
CAGA

Nucleotide sequence of Bacillus subtilis strain H184 is as follows:

GGAACGCGGGCGGCGTGCCTAAT
ACATCCAAGTCGAGCGAATAGAT
TAAGAGCTTGCTCTTATGAAGTTA
GCGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCCATAAGACTG
GGATAACTCCGGGAAACCGGGGC
TAATACCGGATAACATTTTGAACC
GCATGGTTCGAAATTGAAAGGCG
GCTTCGGCTGTCACTTAGATGGAC
CCGCGTCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAAC
GATGCGTAGCCGACCTGGAGGGT
GATCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCA
ACGCCGCGTGAGTGATGAAGGCT
TTCGGGTCGTAAAACTCTGTTGTT
AGGGAAGAACAAGTGCTAGTTGA
ATAAGCTGGCACCTTGACGGTAC
CTAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTATCCG
GAATTATTGGGCGTAAAGCGCGC
GCAGGTGGTTTCTTAAGTCTGATG
TGAAAGCCCACGGCTCAACCGTG
GAGGGTCATTGGAAACTGGGAGA
CTTGAGTGCAGAAGAGGAAAGTG
GAATTCCATGTGTAGCGGTGAAA
TGCGTAGAGATATGGAGGAACAC
CAGTGGCGAAGGCGACTTTCTGG
TCTGTAACTGACACTGAGGCGCG
AAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCC
GTAAACGATGAGTGCTAAGTGTT
AGAGGGTTTCCGCCTTTTAGTGCT
GAAGTTAACGCATTAAGCACTCC
GCCTGGGGAGTACGGCCGCAAGG
CTGAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGAAGCAACGC
GAAGAACCTTACCAGGTCTTGAC
ATCCTCTGACAACCCTAGAGATA
GGGCTTCTCCTTCGGGAGCAGAG
TGACAGGTGGTGCATGGTTGTCGT
CAGCTCGTGTCGTGAGATGTTGGT
TAAGTCCCGCAACGAGCGCAACC
CTTGATCTTAGTTGCCATCATTTA
GTTGGGCACTCTAAGGTGACTGC
CGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGC
CCCTTATGACCTGGGCTACACACG
TGCTACAATGGACGGTACAAAGA

GCTGCAAGACCGCGAGGTGGAGC
TAATCTCATAAAACCGTTCTCAGT
TCGGATTGTAGGCTGCAACTCGCC
TACATGAAGCTGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGG
TGAATACGTTCCCGGGCTTTGTAC
ACACCGCCCGTCACACCACGAGA
GTTTGTAACACCCGAAGTCGGTG
GGGTAACCTTTTTGGAGCCAGCC
GCCTAAGGTGGGACAGATGATTG
GGGTGAAGTCGTAACAAGGTAGC
CGTATGGGAAGGTGT

Effects of Different Glyphosate Concentration on the Growth of Bacillus subtilis H184 and Bacillus cereus BFM4

The growth of B. subtilisH184 and B. cereusBFM4 in different concentrations of glyphosate is shown in Figure 3. The inverse relationship between the growth of B. subtilis and glyphosate shows that the herbicide is toxic to the organisms and this toxicity is concentration dependent. Thus an increase in the concentration of glyphosate brought about a corresponding decrease in the growth of the bacteria isolates for the period of 28 days. The medium containing 20 mg/ml glyphosate, mineral salt medium and B. cereus strain BFM4 was significantly ($P< 0.05$) higher, with the maximum growth of 1.38 (optical density at 482 nm) observed at day 7.

Percentage Utilization of Glyphosate

The percentage utilization in the medium containing 20 mg/ml glyphosate, mineral salt medium (MSM) and Bacillus cereus BFM4 was significantly ($P<0.05$) higher than other treatments, with maximum percentage b utilization of 97.04% at day 28 while the least percentage utilization of 2.15% was observed in the control (30 mg/ml of herbicide +MSM) (Figure 4).

Percentage Utilization of Glyphosate with Heavy Metals Using Bacillus subtilis

The percentage utilization of glyphosate with heavy metals using Bacillus subtilis H184 is shown in Figure 5. The highest percentage utilization (79.02%) of glyphosate by Bacillus subtilis was observed in the medium contaminated with 200 µg/ml Pb^{2+} at day 28, while the least percentage degradation (18.66 %) was observed in the control at day 14.

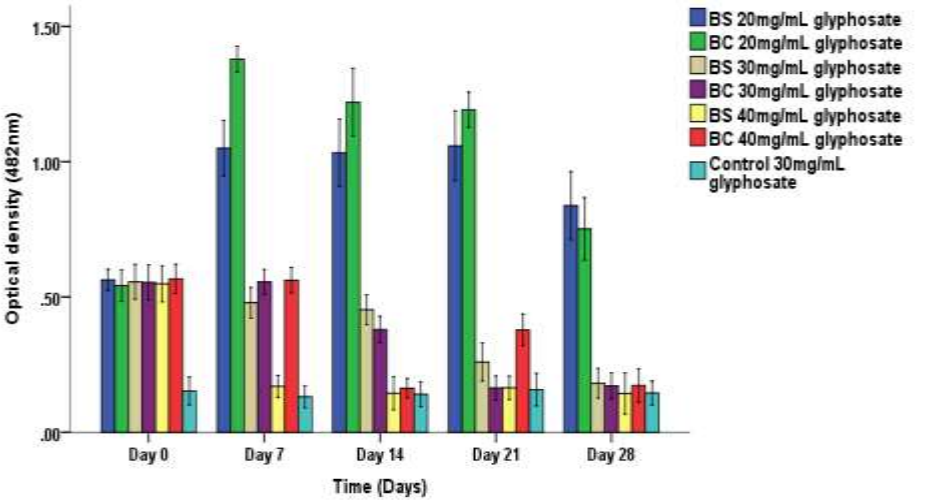

Figure 3: Effects of different glyphosate concentration on the growth of B. subtilisH184and B. cereus BFM4

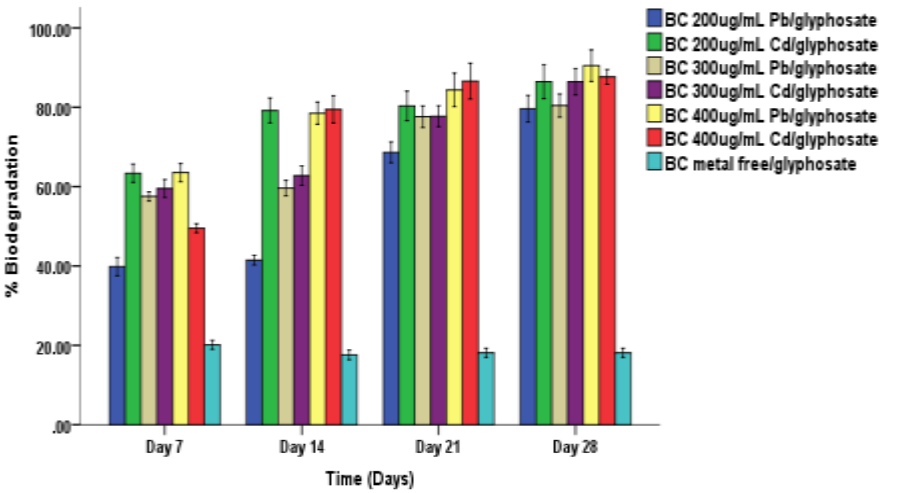

Figure 4: Percentage Utilization of Glyphosate with heavy metals using Bacillus cereusBFM4

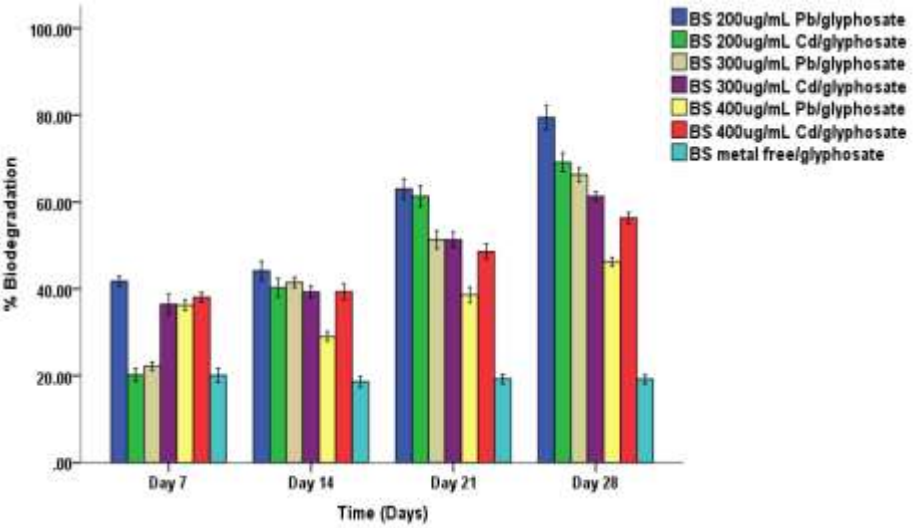

Figure 5: Percentage utilization of glyphosate with heavy metals usingBacillus subtilis H184

Bacterial Count during the Biodegradation of Glyphosate

The highest bacterial count (1.08×10^8 cfu/ml) was observed at 14 days of incubation period in the medium containing Bacillus cereus BFM4 and 20 mg/ml concentration of the herbicide while the least bacterial count (2.47×10^6 cfu/ml) was observed at day 28. The result is shown in Figure 6.

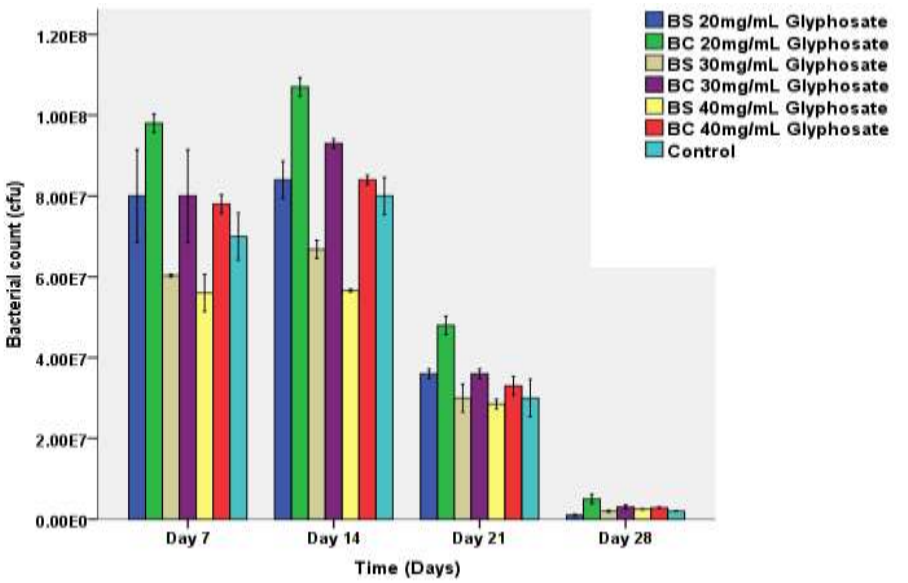

Figure 6: Bacterial Count during the Utilization of Glyphosate

Discussion

Five bacterial isolates were screened for glyphosate utilization by measuring their growth at 482 nm on medium containing glyphosate as sole phosphorous source. Of the five identified bacterial species, two (Bacillus subtilis and Bacillus cereus) were selected for further biodegradation studies based on their short lag phase and rapid utilization of glyphosate (Figure 1). E. coli, Actinomyces sp. and P. aeruginosa did not show appreciable growth as observed in Figure 1. Studies by Baboo et al. [7] showed that culturable bacteria are usually reduced in number or eliminated when extracted from soil or grown on liquid media containing glyphosate, this could be attributed to the effect due to the toxicity of the herbicide. The results in this study, which showed a reduction in the number of bacterial species grown on the glyphosate liquid medium are consistent with the report of Baboo et al. [7].

The growth of B. subtilis H184 and B. cereusBFM4 in different concentration of glyphosate gave an inverse relationship (Figure 3). The medium containing 20 mg/ml glyphosate, mineral salt medium and B. cereus BFM4 was significantly ($P< 0.05$) higher, with the maximum growth of 1.38 (optical density at 482 nm) observed at day 7 (Figure 4). This might be due to the poisonous nature of glyphosate which inhibits growth at higher concentration. This conforms to the findings of PAN International [14] who reported that glyphosate can either stimulate or inhibit soil microorganisms depending on the soil type or herbicide concentration.

The percentage utilization in the medium containing 20 mg/ml glyphosate, mineral salt medium (MSM) and Bacillus cereus BFM4 was significantly ($P< 0.05$) higher than other treatments, with maximum percentage utilization of 97.04% at day 28 (Figure 4) while the least percentage utilization of 2.15% was observed in the control (30 mg/ml of herbicide +MSM). This might be because the low concentration of the herbicide served as a substrate to the bacteria. This agrees with the findings of Huang et al.[9] who revealed that up to 20-90% of glyphosate was mineralized to CO_2 by bacteria over approximately 5 weeks, depending on the soil type.

The percentage utilization was significantly ($P < 0.05$) higher (90.49%) in the medium containing B. cereus BFM4 with herbicide contaminated with 400 µg/ml of Pb^{2+} while the least percentage utilization (18.12%) was observed in the control (metal free) as seen in Figure 5. Bacillus cereusstrain BFM4 could tolerate high heavy metal concentration. Sevim and Sevim, [15] and Adekanmbi [16] reported that heavy metal resistance in Bacillus sp. is mostly mediated by plasmids that they possess. Statistical analysis revealed that there was significant ($P< 0.05$) difference in the treatments at day 28 (Figure 5), with the medium containing B.subtilis H184 with herbicide contaminated with 200 µg/ml Pb^{2+} being significantly higher than the other treatment with a maximum percentage glyphosate utilization of 79.02 %, this could be attributed to large ionic size and heavier atomic weight of lead compared to cadmium, which enables it to have greater interaction with biological components.

This result is in line with the findings of Shameer [17] and Shamim [18] who reported that large ionic size of lead makes it to be adsorbed more than cadmium by Bacillus sp. The highest bacterial count (1.08×10^8 cfu/ml) was observed at 14 days of incubation period

in the medium containing Bacillus cereusBFM4 and 20 mg/ml of the herbicide as observed in Figure 6. The increase in bacterial count observed at day 14 in the medium containing Bacillus cereusBFM4 and 20mg/ml of the herbicide might be due to the fact that the organisms were able to utilize the herbicide as a source of nutrient for their growth and metabolism. The reduction in the bacterial count at day 21 and 28, could be attributed to the depletion of nutrients in the medium due to bacterial utilization. This is in agreement with the findings of Torretta et al. [8] who revealed that nutrient availability affects bacterial counts during biodegradation of herbicides.

Conclusion

Bacillus cereus BFM4 and Bacillus subtilis H184 were able to utilize glyphosate as sole carbon and phosphorus sources. Bacillus cereus BFM4 had higher percentage glyphosate utilization of 97.04 % in glyphosate at the lowest concentration of the herbicides. However, Bacillus cereus BFM4 had higher percentage utilization of 90.49 % in glyphosate at concentration (400 µg/ml) of the heavy metal (lead). The addition of trace amount (200 µg/ml, 300 µg/ml and 400 µg/ml) of heavy metals stimulated microbial growth, hence increasing the rate by which he glyphosate utilized the herbicides.

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Faith Onyinyechi Mbagwu, et al *CJPL (2019) 7(2) 10-23*