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Original Paper

Biodegradation of Glyphosate by Bacteria Isolated from Agricultural Fields

OnuorahSamuelChinedu*, Obiefuna HelenObianuju

Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria.

**Corresponding Author email: s_onuorah484@yahoo.com*

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The most commonly used herbicide around the world is glyphosate, mostly sold as "Roundup". Due to its intensive use both in agricultural and non-agricultural purposes which has led to its accumulation in the soil, it has become a matter of environmental concern and the best option to ease its effects is through bioremediation. Four bacterial strains (Exiguobacterium alkaliphiluim, Alcaligenes faecalis, Sinorhizobuim fredi and Acinetobacter nosocomialis.) were isolated from three different agricultural fields polluted with glyphosate. The ability of the four strains to degrade glyphosate as a sole source of carbon, singly and as a consortium was evaluated in this study. Their degradation abilities were evaluated by checking the residual glyphosate after 14 days' incubation, using Gas Chromatography – Mass Spectrophotometer (GC-MS). Their glyphosate degradation capability in mineral salt medium with the addition of glucose and ammonium sulphate was analyzed separately and as a consortium. At the end of the 14-day incubation period the residual glyphosate was far lower when pesticide was used as a sole source of carbon than when glucose or ammonium sulphate or both were added to the MSM. It was observed that the consortium had a better degradation ability of glyphosate. Based on these results the four bacterial strains do not require bio stimulation to mineralize glyphosate, thus these bacteria can be used for remediation of glyphosate contaminated soil.

Keywords: Glyphosphate herbicide, degredation

INTRODUCTION

Herbicides are by far the most commonly used pesticides. They range from non-selective to highly selective for control of specific weeds in specific crops, with different products having post- emergence, pre-plant and pre-emergence use. More than 500 different pesticide formulations are being used in the environment, mostly in agriculture [1]. The intensive use ofherbicide is a general practice and thus a matter of environmental concern. This is as a result of the potential hazardous effects of the chemicals on soil biological processes, non- target organisms and pollution of streams and rivers through runoffs [2]. Pesticides have significant chronic human health effects including cancer, neurological effects, diabetes, respiratory diseases, fetal disease and genetic disorder [3,4].

The most commonly used herbicide around the world is glyphosate, mostly sold as "Roundup" (brand name). Glyphosate (Isopropylamine salt of N-Phosphono-methyl-glycine) is a broad-spectrum systemic, post- emergence, non-selective herbicide that inhibits the

enzyme 5-enolpyruvicshikimic acid-3-phosphate synthase (EPSPS), blocking the synthesis of essential aromatic amino acids [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-phosphorous bond) present in the compound [6,7]. Chemical processes are also considered expensive since it is difficult to come up with a single method that can remove herbicides from soil, water and waste water [8,9]. Biodegradation still remains the most favoured option. Many bacteria that are able to degrade glyphosate have been isolated from soil around the world [10], thus the aim of this study was to
isolate the bacteria present in agricultural fields that are capable of degrading glyphosate and to evaluate the effect of bio stimulation with inorganic substrates on the degradation abilities of the isolates, singly and as a consortium.

MATERIALS AND METHODS

Chemicals and media

The isopropylamine salt of glyphosate known as Roundup® (containing 450g active ingredient/L of glyphosate) was purchased from Agricultural Department Programme (ADP) Awka, Anambra State, Nigeria. Soil samples were collected from three different agricultural fields treated with glyphosate in Awka, Anambra State, Nigeria.
Mineral Salts Medium (MSM) was used for the isolation of bacteria using glyphosate as a sole carbon and energy source. The MSM contained in gram per liter of distilled water: KH_2PO_4 (1.5), Na_2HPO_4 (0.6), NaCl (0.5), NH_4SO_4(2), $CaCl_2$(0.01), $MgSO_4.7H_2O$ (0.2), $FeSO_4.7H_2O$(0.001).

Collection of soil samples

Glyphosate-contaminated soil samples were collected from three farms within Awka. Each farm was divided into 5 portions and triplicate soil cores obtained from each portion from depth 0-15cm, which were pooled, sieved, homogenized and immediately transferred to the laboratory using sterile transport polyethylene bag.

Isolation of glyphosate degrading bacteria

This was done according to the method described by Benslama and Boulhahrauf 2013 [11]. A 5.0g portion of the homogenized polluted soil sample was added to 95ml of mineral salts medium (MSM) in 250 ml flasks with the addition of 1ml of glyphosate and incubated at 30°C in a rotary shaker (150rpm) for seven days. Thereafter, 1ml was collected from the flask and diluted ten-fold in sterile distilled water. A 1 ml aliquot of 10^{-2} dilution factor was pour plated on mineral salts agar plates containing 1ml glyphosate. The plates were incubated for 72h at room temperature. Afterwards, colonies that developed on the plates were counted, picked and sub-cultured on fresh nutrient agar plates to obtain pure cultures. Pure cultures were stored on nutrient agar slants.

Characterization and identification of bacterial isolates.

The bacterial isolates obtained were characterized and identified on the basis of morphological-and biochemical characteristics using the methods described by Cheesbrough, 2006 [12] Fawole, 2001 [13]. Further identification of the bacterial isolates was done using the 16SrRNA gene extraction, PCR and sequencing. The 16S rRNA gene of the isolates was amplified using the PCR universal primer; 27F:5'- AGAGTTTGATCGTGGCTCAG-3and

5’-1492R;TACGGTTACCTTGTTACGACTT-3’, which corresponds to the forward and reverse primers of 16SrRNA.

Degradation by mono and mixed cultures

This was done according to the method described by Benslama and Boulahrauf, 2013[11]. Each of the bacterial isolates was inoculated into a separate 99ml of MSM containing 1ml of glyphosate in 250ml flask. All the isolates were also inoculated into one 250 ml conical flask containing 1ml of glyphosate in 99 ml of MSM and incubated alongside the monocultures for 14 days in a rotary shaker at 150 rpm at 30^{o}C.

This procedure was repeated with 1g/L glucose added, 1g/L ammonium sulphate added and also repeated with 1g/Leach of glucose and nitrogen source (ammonium sulphate) added together into the MSM medium. After 14 days’ incubation the residual glyphosate was determined using GC-MS.

RESULTS AND DISCUSSION

Identification of isolated strains

Identification of the bacteria isolated from the contaminated soil samples that were able to utilize glyphosate, using 16S rRNA typing revealed the presence of *Exiguobacterium alkaliphilum, Alcaligenes faecalis, Sinorhizobium fredii* and *Acinetobacter nosocomialis* (Table 1)

Acinetobacter sp. has been reported by Olawale and Akintobi, 2011 [14] as a glyphosate degrader. “Krychukova et al; 2014 [15] reported the presence of *Alcaligenes* in glyphosate contaminated soil” and Mussali- Galante et. al; 2021 [16] also reported the presence of *Sinorhizobium sp*. *Exiguobacterium alkaliphilum* has been reported as a good crude oil and beverage effluent degrader [17]. However, this finding has also shown that this bacterium is a good glyphosate pesticide degrader. *Sinorhizobium fredii* is a known nitrogen fixing bacteria [18], thus, its ability to survive the presence of glyphosate, could be deduced to be as a result of the fact that glyphosate pesticide is a nitrogen-based chemical. *Acinetobacter nosocomialis* is a well-known opportunistic pathogen that is usually implicated in acquired hospital infections [19]. Its presence in the soil sample used for glyphosate degradation study could be as a result of the fact that one of the examined farmlands was sited closely to a hospital. This however, showed that some hospital-associated strains of microorganisms end up being found in the surrounding environment, most likely through improper hospital waste disposal to the environment; perhaps without prior waste treatment. This finding showed that *Acinetobacter nosocomialis* is not just a known hospital opportunistic pathogen, but also a glyphosate degrader.

Table 1: Molecular Identification of Glyphosate Utilizing Bacteria

Bacteria	Molecular Sequence ID
Exiguobacterium alkaliphilum	CP073101.1
Alcaligenes faecalis	MF510848.1
Sinorhizobium fredii	AB825994.1
Acinetobacter nosocomialis	MH368653.1

Bio stimulation with inorganic substrate

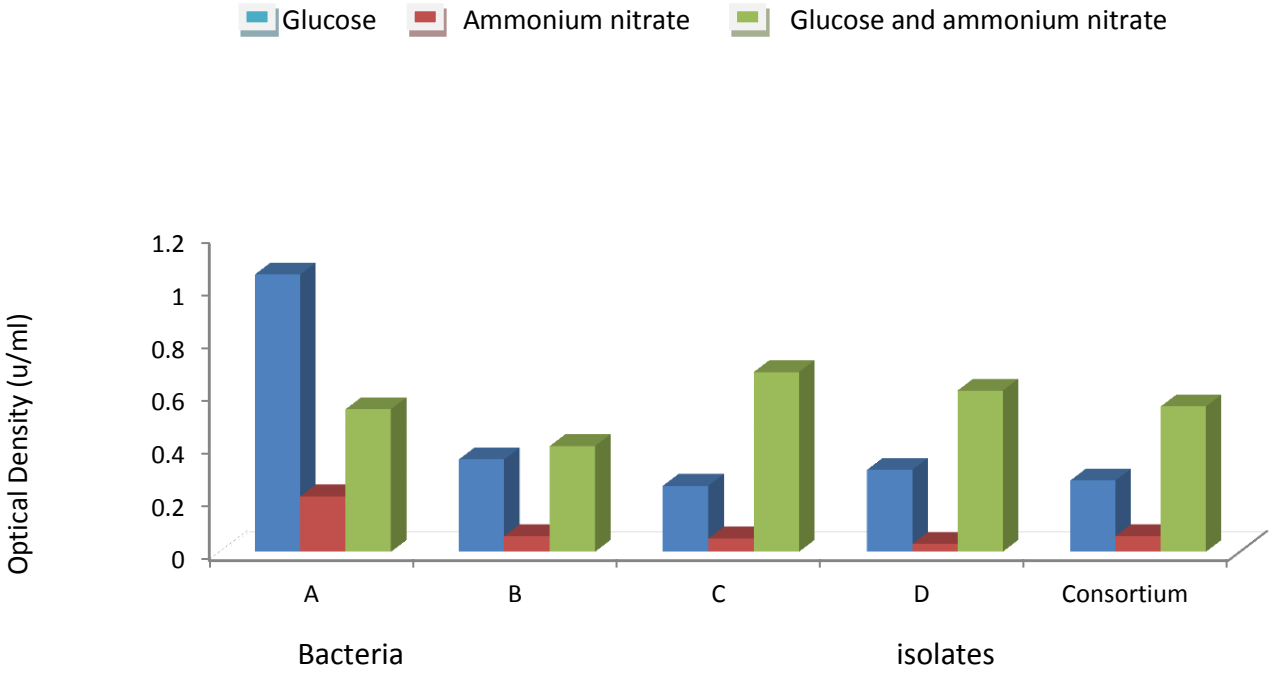
The impact of bio-stimulation on the capacity of the isolated bacteria to degrade glyphosate was determined. Glucose and ammonium sulphate were employed as the rate limiting nutrient to check if they could stimulate microbial biomass growth and enhance glyphosate degradation. Employment of bio-stimulation strategy to enhance glyphosate degradation has been reported by Ramdas and Sims, 2011 [20]. It was observed in this study that the degraders and their consortium had notable biomass growth with the incorporation of glucose and the incorporation of a combination of glucose and ammonium sulphate. They however, did not show notable biomass growth with the incorporation of ammonium sulphate alone, except for *Exiguobacterium alkaliphilum.* (Fig. 1).

Aside biomass growth, this bio-stimulation strategy also brought about some notable points: All the organisms which originally degraded glyphosate as a sole source of carbon, (Table 2) apparently lost the ability to relatively degrade the pesticide with the incorporation of glucose (Table3).

All the organisms also could not achieve good degradation with the incorporation of ammonium sulphate alone (Table 4).

However, the value of residual glyphosate was less with ammonium sulphate incorporation than with glucose incorporation.

These occurrences call to mind, the possible reasons why the strategy of bio-stimulation, could not achieve expected mineralization at short time. According to [21], two main reasons for these observations may be enzyme-mediated metabolism and chemical kinetics of the degradation reaction. This study showed that bio stimulation with inorganic substrate did not enhance the degradation of glyphosate within the experimental period.



A: *Exiguobacterium alkaliphilum* B: *Alcaligenes faecalis* C: *Sinorhizobium fredii* D: *Acinetobacter nosocomialis*

Figure 1: Effect of Different Substrates on Microbial Growth in Glyphosate

Table 2: GC-MS result for Glyphosate degradation by isolates without bio stimulation

Isolates	Residual-Glyphosate Concentration (ug/ml)	Percentage Degradation
Exiguobacterium alkaliphilum	2.81	97.36
Alcaligenes faecalis	2.61	97.55
Sinorhizobium fredii	3.38	96.82
Acinetobacter nosocomialis	2.99	97.19
Consortium	2.57	97.58

Table 3: Effect of Glucose (1ml) Substrate on Glyphosate Degradation

Isolates	Residual-Glyphosate Concentration (ug/ml)	Percentage Degradation
Exiguobacterium alkaliphilum	12.41	88.44
Alcaligenes faecalis	18.07	83.05
Sinorhizobium fredii	15.24	85.64
Acinetobacter nosocomialis	17.87	83.16
Consortium	9.94	90.63

Table 4: Effect of Ammonium sulphate (1ml) Substrate on Glyphosate Degradation

Isolates	Residual-Glyphosate Concentration (ug/ml)	Percentage Degradation
Exiguobacterium alkaliphilum	7.70	92.74
Alcaligenes faecalis	8.29	92.19
Sinorhizobium fredii	12.64	88.09
Acinetobacter nosocomialis	6.00	94.34
Consortium	5.98	94.36

Table 5: Effect of Glucose and Ammonium sulphate (1ml) on Glyphosate Degradation

Isolates	Residual-Glyphosate Concentration (ug/ml)	Percentage Degradation
Exiguobacterium alkaliphilum	6.73	93.66
Alcaligenes faecalis	6.65	93.73
Sinorhizobium fredii	9.66	90.90
Acinetobacter nosocomialis	8.36	92.12
Consortium	5.05	95.24

CONCLUSION.

This study reports the isolation and identification of four bacterial strains *Exiguobacterium alkaliphiluim, Alcaligenes faecalis, Sinorhizobuim fredi* and *Acinetobacter nosocomialis* from agricultural fields in Awka Anambra State Nigeria that possess the capacity to degrade glyphosate. The application of these bacteria and their consortium can be used to remediate soil contaminated with pesticide. This work also shows that these bacterial strains does not need bio stimulation for the mineralization of glyphosate.

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CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest of any sort with this study.

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