

Full Length Research Paper

Biodegradation of glyphosate herbicide *in vitro* using bacterial isolates from four rice fields

A. N. Moneke*, G. N. Okpala and C. U. Anyanwu

Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.

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Glyphosate is a compound used as herbicide in the control and/or killing of grasses and herbaceous plants. It can be used in no-till 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 contaminated soils and bioremediation is a very useful alternative to conventional cleanup methods. The success of this will depend on isolating bacteria with the ability to degrade glyphosate in a changing environment. The abilities of five bacterial species (*Escherichia* sp., *Azotobacter* sp., *Alcaligenes* sp., *Acetobacter* sp. and *Pseudomonas fluorescens*) 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 and/or phosphorus source. Of the five bacterial isolates, *P. fluorescens* and *Acetobacter* sp. showed the capacity to utilize glyphosate efficiently and were therefore used for further biodegradation studies. Time course of growth of the isolates on mineral salt medium containing glyphosate showed that both grew significantly ($P < 0.05$). Microbial growth during the study was monitored by measuring the optical density at 660 nm. The comparative effects of glyphosate as carbon and/or phosphorus source on the growth of the isolates showed that there was significant ($P < 0.05$) growth in the medium containing glucose and glyphosate. The effects of different concentrations of glyphosate on the growth of the isolates (*P. fluorescens* and *Acetobacter* sp) were evaluated. Significant ($P < 0.05$) growth was observed at lower concentrations (7.2 - 25 mg/ml) of glyphosate. No inhibition of growth was observed at high concentrations (100 - 250 mg/ml), indicating that the isolated bacteria can tolerate up to 250 mg/ml of glyphosate. However, there was subsequent decrease in growth of both isolates as the concentration of glyphosate increased. This study showed that *P. fluorescens* and *Acetobacter* sp. exhibited a high capacity to efficiently degrade glyphosate under the environmental conditions studied. Thus, the organisms can be exploited for biodegradation of glyphosate and should be studied for their ability to degrade other organophosphates.

Key words: Glyphosphate herbicide, degradation process.

INTRODUCTION

The intensive use of herbicides in rice-based 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-dichlorophenoxyacetic acid (2,4-D) and Roundup® (isopropylamine salt of glyphosate). 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 (Atkinson, 1985). Also, 2,4-dichlorophenoxy-acetic acid which is used by most farmers to spike glyphosate in order to boost its efficacy increases the toxicity of glyphosate.

*Corresponding author. E-mail: annymoneke@yahoo.com. Tel: 08033357734.

Table 1. State and location from where the soil samples were collected.

State	Location	Code
Anambra	Omor	OMR
Anambra	Omasi	OMS
Enugu	Adani	ADA
Ebonyi	Abakaliki	AKL

Organophosphates, including glyphosate, account for half of the pesticides used worldwide with glyphosate based formulations such as Roundup®, Accord® and Touchdown® consisting the commonest types used for agricultural purposes (Franz et al., 1997). Glyphosate is a broad spectrum, non-selective herbicide used in the control and/or killing of grasses, herbaceous plants, including deep rooted perennial weeds, brush, some broad-leaf trees and some shrubs (United States Department of Agriculture (USDA), 2000; Cox, 2000). It can be used in no-till agriculture, to prepare fields before planting, during crop development and after crop harvest (USDA, 2000). Its mode of action is the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase, resulting in the depletion of essential aromatic amino acids needed for plant survival (Arhens, 1994; Zablotowicz and Reddy, 2004). 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 (Cox, 2000; Santillo et al., 1989). 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 (Penalzoza-Vazquez et al., 1995). 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 (Gimsing et al., 2004). Glyphosate is microbially degraded in soil and water and has a reported field half-life of 47 days and a laboratory half-life of < 25 days (Ahrens, 1994). 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. Microorganisms known for their ability to degrade glyphosate in soil and water include *Pseudomonas sp* strain LBr (Jacob et al., 1988), *Pseudomonas fluorescens* (Zboinska et al., 1992), *Arthrobacter atrocyaneus* (Pipke et al., 1988) and *Flavobacterium sp.* (Balthazor and Hallas, 1986). Bacteria degrade glyphosate via two general pathways leading to the intermediate production of either glycine or aminomethylphosphonate (AMPA). The use of glyphosate

based formulations in rice farming is a common practice in south eastern Nigeria and in oil palm (*Elaeis guineensis*) plantation in the rainforest area of Nigeria. No work has been done to identify glyphosate degrading bacteria from rice farms in Nigeria and the conditions that enhance the degradation process. The main objectives of the present study are the isolation and identification of glyphosate utilizing bacteria from rice farms using an enrichment culture technique, assessment of the growth response of the isolates in liquid medium containing different concentrations of glyphosate and analysis of the comparative effects of glyphosate as carbon or phosphorus source on the isolates.

MATERIALS AND METHODS

Chemicals used

The isopropylamine salt of glyphosate known as Roundup® (containing 360 g active ingredient/L of glyphosate, Monsanto) was purchased from a local dealer's store in Nsukka, Enugu state, Nigeria. All other chemicals were of the highest purity commercially available.

Collection of soil samples

Soil samples were obtained from four rice fields located at Adani in Enugu State, Nigeria, Omor and Omasi in Anambra State, Nigeria and Abakaliki in Ebonyi State, Nigeria, all in south eastern Nigeria. These rice fields are known to have been previously exposed to glyphosate-based formulation (Roundup®) for long periods of time. Soil samples were collected from depths of 0 - 15 cm from three different sites in each of the four locations. Soil samples from each site were thoroughly mixed. They were designated as shown in Table 1. All samples were placed in sterile polyethylene bags and taken immediately to the laboratory and stored at 4°C before use within 24 h (Nannipieri, 1994).

Isolation medium

A modified mineral salts medium (MSM) of Dworkin and Foster (1958) consisting of (g/l) (NH₄)₂SO₄, 0.375; MgSO₄, 0.075; CaCO₃, 0.03; FeSO₄.7H₂O, 0.001; H₃BO₃, 0.000001, MnSO₄, 0.000001 and yeast extract, 0.0053 was used. Phosphate buffer was replaced by tris buffer 6.05 g/L and pH was adjusted to 7.0. All glasswares were washed with 1 N HCl and thoroughly rinsed with deionized water to remove contaminating phosphate before use. The medium was autoclaved at 121°C and 15 psi for 15 min prior to the addition of the filter sterilized Roundup® (isopropylamine salt of glyphosate) and glucose (1.0) autoclaved at 110°C and 10 psi as carbon source.

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 Roundup® (7.2 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 (Gallenkamp, England) at 120 rpm for 7 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 7 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 (Fluka). Identity of the isolates was affirmed after characterization by standard bacteriological methods (Holt et al., 1994; Cheesbrough, 1984). Stock cultures were maintained in nutrient agar slants at 4°C.

Inoculum preparation and standardization

Inocula used for the study were prepared by inoculating isolates into nutrient broth and incubated at 30°C for 24 h using sterile normal saline; the cells from the above cultures were resuspended to a 0.5 McFarland nephelometer standard (Optical density of 0.17 at 660 nm).

Glyphosate utilization patterns of the different isolates

A 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 without yeast extract. It contained 3 ml of roundup (7.2 mg/ml of glyphosate). The flasks were incubated on a rotary shaker (Gallenkamp, England) at 120 rpm for 180 h at 30°C. The ability of each isolate to utilize glyphosate was measured based on the turbidity of the medium at 660 nm using a spectrophotometer (Spectronic 20, USA).

Determination of time course of the growth of *P. fluorescens* and *Acetobacter* sp.

500-ml Erlenmeyer flasks containing 150 ml of the sterile screening medium was prepared and 3 ml of roundup (containing 7.2 mg/ml of glyphosate) was added to each flask. 1 ml of the inoculum (0.5 Macfarlane standards) 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 patterns. The medium was then incubated at 30°C for 192 h 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.

Comparative role of glyphosate as carbon or phosphorus source

The screening medium (150 ml) was prepared as earlier described and 3.0 ml filter-sterilized roundup (containing 7.2 mg/ml of glyphosate) was added as phosphorus or carbon source. When used as carbon source, denoted by Gly and Pi, the medium consisted of the following (g/L): $(\text{NH}_4)_2\text{SO}_4$, 0.375; MgSO_4 , 0.075; CaCO_3 , 0.03; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; H_3BO_3 , 0.000001; MnSO_4 , 0.000001; NaH_2PO_4 , 6.0 and KH_2PO_4 , 2.0. When used as carbon and phosphorus source, denoted by (Glyphosate), the medium consisted of the following (g/L): $(\text{NH}_4)_2\text{SO}_4$, 0.375; MgSO_4 , 0.075; CaCO_3 , 0.03; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; H_3BO_3 , 0.000001; MnSO_4 , 0.000001; tris buffer 6.05 g. When used as phosphorus source denoted by (Gly and Glu), the medium consisted of the following (g/L): $(\text{NH}_4)_2\text{SO}_4$, 0.375; MgSO_4 , 0.075; CaCO_3 , 0.03; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$,

0.001; H_3BO_3 , 0.000001; MnSO_4 , 0.000001; glucose, 1.0; tris buffer 6.05 g. The media was incubated at 30°C for 120 h 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.

Effects of different concentrations of glyphosate on the growth of the isolates

Aliquots (1.0 ml) of 24 h old bacterial cultures (0.5 MacFarland standard) grown in nutrient broth were inoculated into 500-ml Erlenmeyer flasks containing 150 ml of MSM supplemented with various concentrations of glyphosate (25, 50, 100 and 250 mg/ml). A control was maintained with MSM supplemented with 7.2 mg/ml of glyphosate. Bacterial growth was monitored by withdrawal of 5.0 ml of culture sample immediately after inoculation for the 0 h and every 12 h up to 108 h of incubation and optical density was measured at 660 nm.

Treatment effects on the growth of the bacterial isolates at the different time periods were analysed using 2-way ANOVA.

RESULTS

Isolation of glyphosate utilizing bacteria

The preliminary studies with glyphosate as phosphorus source showed that a total of twelve bacterial isolates were able to grow in the presence of glyphosate as sole phosphorus source. On further sub-culturing on solid medium, five isolates consistently grew on the MSM enriched with glyphosate as phosphorus source. They are: *Acetobacter* sp. - G ADA3, *Escherichia* sp. - G AKL2, *P. fluorescens* - G AKL5, *Azotobacter* sp. - G OMR1 and *Alcaligenes* sp. - G OMS1.

Glyphosate utilization patterns of the different isolates

The five bacterial isolates were screened for glyphosate utilization by measuring their growth turbidimetrically at 660 nm. Of the five bacterial isolates grown on the medium containing glyphosate as sole phosphorus source, *P. fluorescens* significantly ($P < 0.05$) utilized glyphosate (mean OD 0.1268). This was followed by *Acetobacter* sp., *Azotobacter* sp. and *Alcaligenes* sp. (mean OD 0.1069, 0.0858 and 0.0841, respectively). *Escherichia* sp. did not show any appreciable growth as shown in Figure 1.

Growth kinetics of *P. fluorescens* and *Acetobacter* sp. in glyphosate

The growth kinetics of *P. fluorescens* and *Acetobacter* sp. were further monitored over time at 660 nm, using the MSM enriched with glyphosate as sole phosphorus source. Their growths were both significant ($P < 0.001$), but that

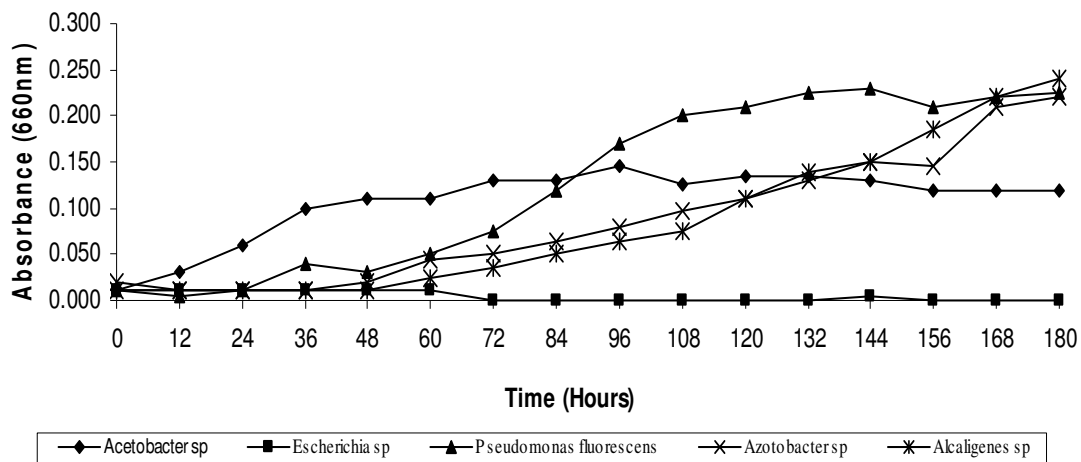


Figure 1. Screening of the isolates for glyphosate utilization.

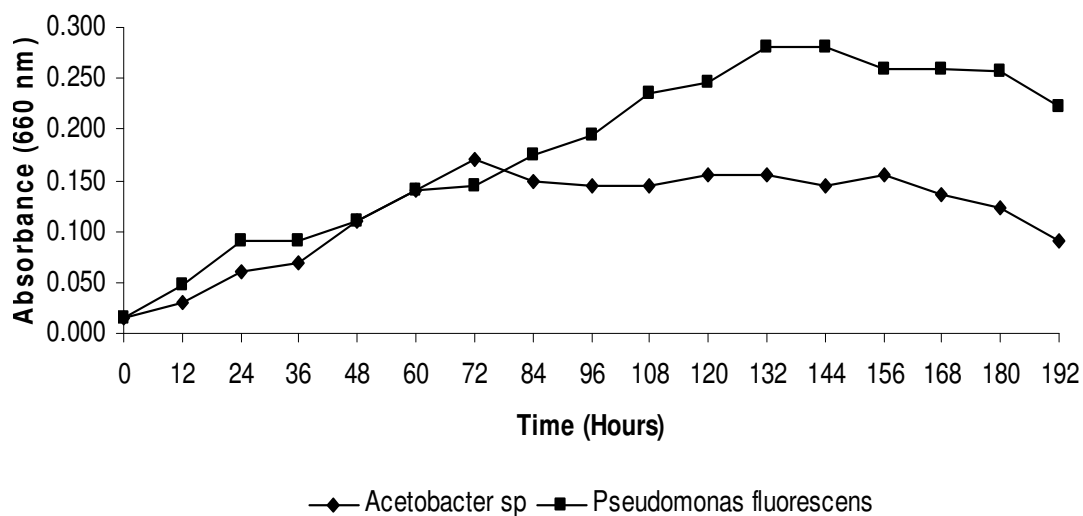


Figure 2. Growth kinetics of *Acetobacter* sp. and *P. fluorescens* on glyphosate.

but that of *P. fluorescens* was significantly ($P < 0.05$) higher than that of *Acetobacter* sp. as shown in Figure 2.

Comparative role of glyphosate as carbon or phosphorus source

The ability of glyphosate to serve as carbon source, phosphorus source, carbon and phosphorus source was monitored. Optical density measurement at 660 nm was used to monitor increase in cell numbers. The growth of *Acetobacter* sp. was non-significantly ($P < 0.05$) higher in the Glucose and Glyphosate (Gly and Glu) medium (mean OD value = 0.0907) when compared to *P. fluorescens* (mean OD value = 0.09003) (Figure 3). On the medium with glyphosate as carbon and phosphorus

source, the growth of *P. fluorescens* was significantly higher when compared to *Acetobacter* sp. The growth kinetics of the isolates in the different carbon sources showed that there was progressive increase in growth of the isolates when glyphosate was used as a phosphorus source and glucose as carbon source. The growth of *Acetobacter* sp. after 24 h incubation on Glu and Gly medium was more significant ($P < 0.05$) with a mean OD value of 0.0933 when compared with the growth on the GPi, glyphosate and the control (Figure 4). Also, the growth of *Acetobacter* sp. on the GPi medium peaked after 36 h with mean OD value of 0.02733, which was significantly ($P < 0.05$) higher than growth on the medium containing glyphosate and the control. After 36 h of incubation, the growth on the Glu and Gly medium consistently increased till the end of the monitoring (120

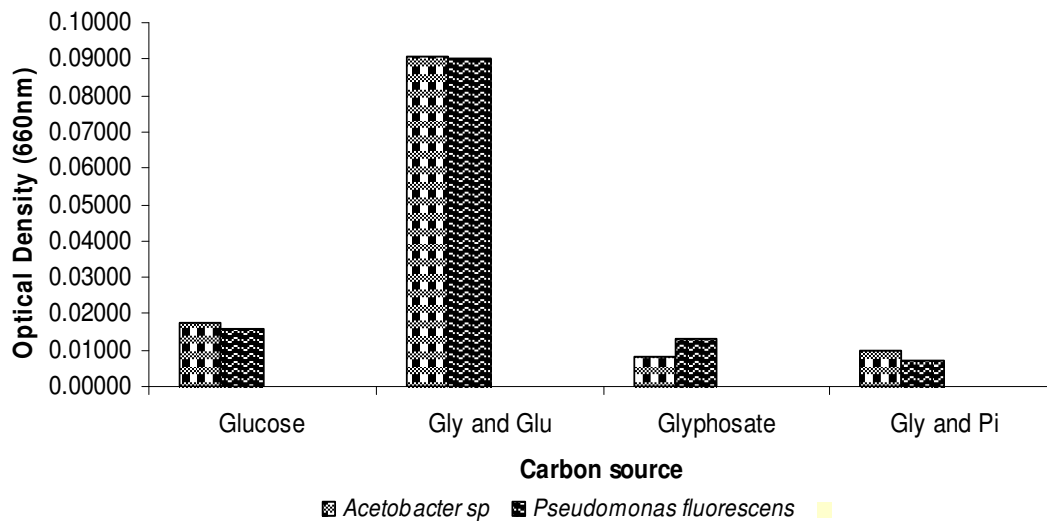


Figure 3. Comparative effect of glyphosate as carbon or phosphorus source on *Acetobacter sp.* and *P. fluorescens*.

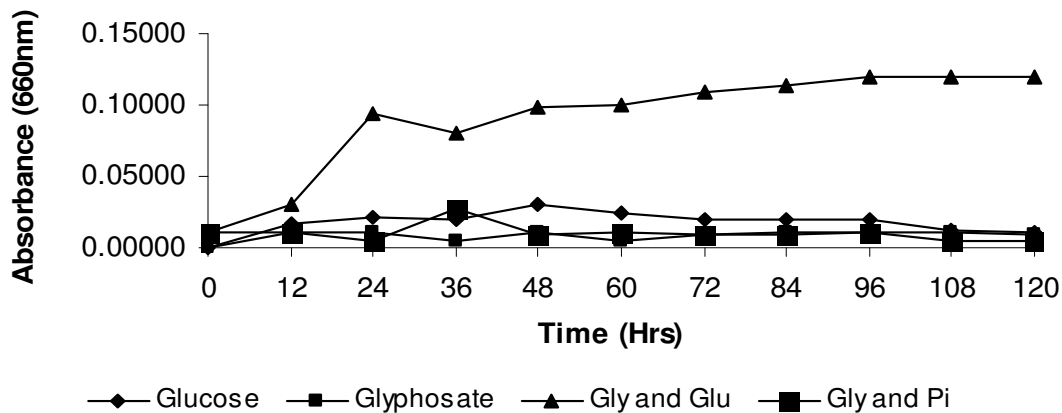


Figure 4. Growth kinetics of *Acetobacter sp.* in glyphosate as carbon or phosphorus source.

h). The growth of *P. fluorescens* in the Glu and Gly medium followed a similar pattern as that of *Acetobacter sp.* as shown in Figure 5.

Effects of different glyphosate concentration on the growth of *Acetobacter sp.* and *P. fluorescens*

The growth of *Acetobacter sp.* and *P. fluorescens* in different concentrations of glyphosate gave an inverse result as shown in Figure 6. As the concentration of glyphosate increased there was a corresponding decrease in the growth of the isolates. The highest growth was observed in the control (7.2 mg/ml) which contained the least concentration of glyphosate. The growth kinetics of both isolates in increasing concentrations of glyphosate followed a similar pattern

with a lag phase of about 12 h and steady increase in growth as seen in Figures 7 and 8. After 84 h of incubation the growth of *P. fluorescens* in medium containing 25 mg/ml of glyphosate increased significantly ($P < 0.05$) when compared with its growth in the medium with 7.2 mg/ml of glyphosate till the end of the monitoring at 108 h.

DISCUSSION

Twelve bacterial isolates were initially isolated from rice field soil samples. On further sub-culturing on solid media enriched with glyphosate, only five showed the capacity to grow in the presence of the herbicide. The five bacterial isolates were identified as *Acetobacter sp.*, *Escherichia sp.*, *P. fluorescens*, *Azotobacter sp.*, and

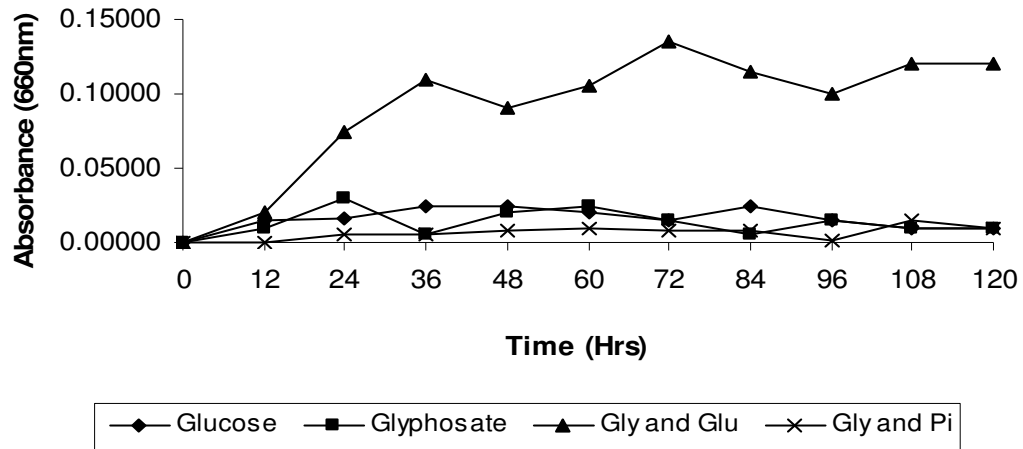


Figure 5. Growth kinetics of *P. fluorescens* in glyphosate as carbon or phosphorus source.

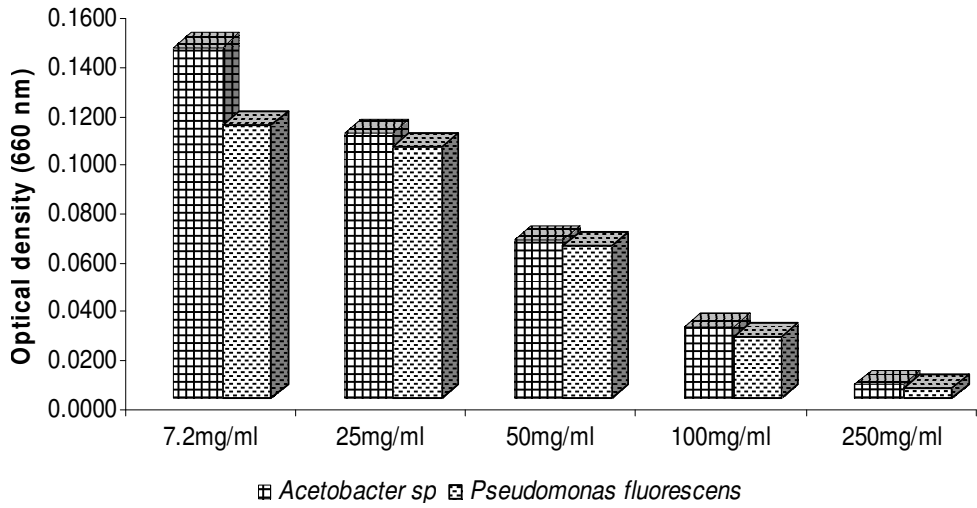


Figure 6. Effects of the different concentrations of glyphosate on the growth of *Acetobacter sp.* and *P. fluorescens*.

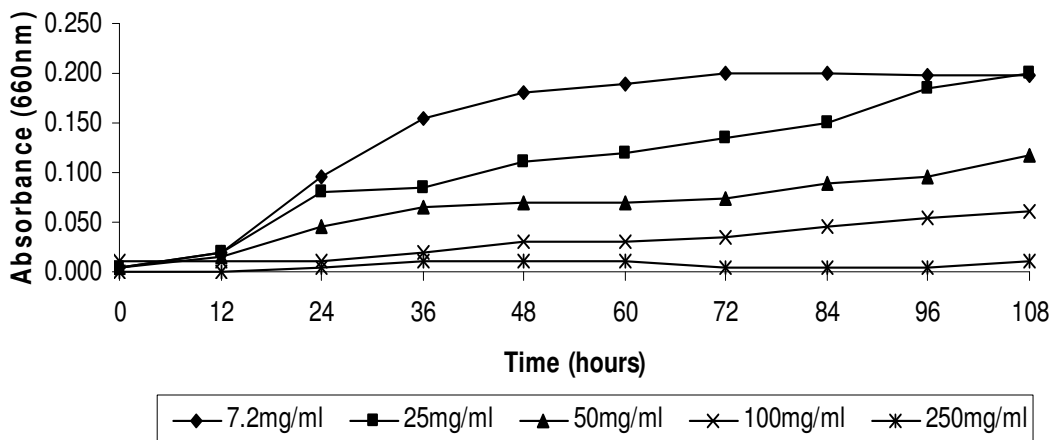


Figure 7. Growth kinetics of *Acetobacter sp.* on the different concentrations of glyphosate.

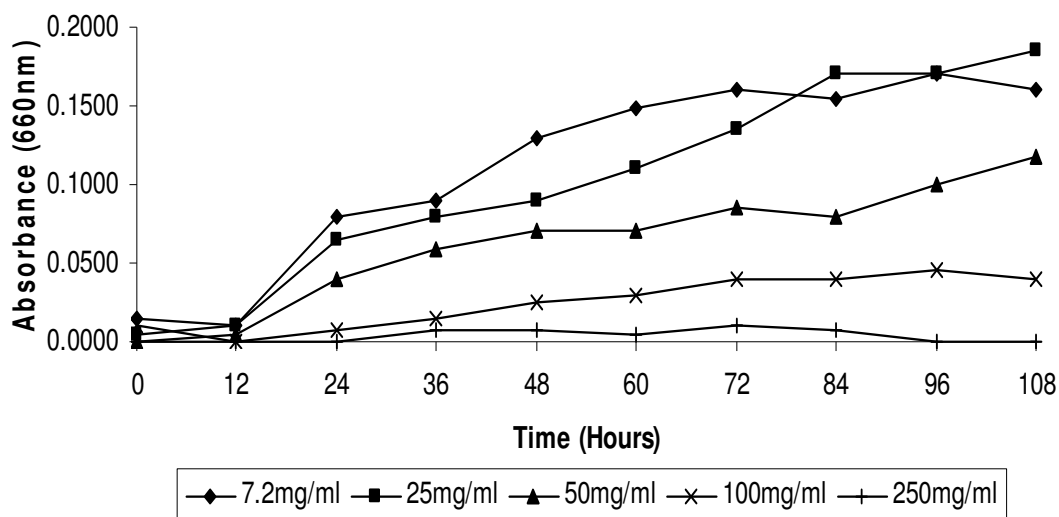


Figure 8. Growth kinetics of *P. fluorescens* on the different concentrations of glyphosate.

Alcaligenes sp. The results of this study, which showed a reduction in the number of bacterial species grown on glyphosate solid medium, are consistent with the report of Busse et al. (2001) who showed that culturable bacteria and fungi are usually reduced in number or eliminated when extracted from soil or grown on solid media containing glyphosate. Other studies (Quinn et al., 1988; Santos and Flores, 1995; Kryzsko-Lupicka and Orlik, 1997) had found similar reductions in population counts when glyphosate was added to culture media. Toxicity of the artificial media is expected to be based on the mode of action of glyphosate (inability of the organism to synthesize the needed aromatic amino acids). Unlike the response in artificial media, no toxicity was expressed when glyphosate was added to soil in laboratory bioassays (Busse et al., 2001).

The isolated bacterial species had previously been obtained from other soil samples (Zboinska et al., 1992, Franz et al., 1997). Of the seven identified bacterial species, two (*Acetobacter* sp. and *P. fluorescens*) were selected for further biodegradation studies based on their short lag phase and rapid utilization of glyphosate. Many *Pseudomonas* species have been used extensively in the degradation and/or metabolism of glyphosate (Jacob et al., 1988; Shinabarger et al., 1984; Kishore and Jacob, 1987; Talbot et al., 1984). However, Zboinska et al. (1992) reported that *P. fluorescens* could not utilize glyphosate. The strain of *P. fluorescens* used in this study was not only able to utilize glyphosate but was also able to thrive at high concentrations of the herbicide. The use of *Acetobacter* in the degradation/metabolism of glyphosate has not been reported.

Both organisms used in this study showed appreciable growth in the culture medium containing glyphosate as sole phosphorus source. The differences observed in the

growth of the isolates in the medium are indicative of the differences between the organisms in tolerating the herbicide. The short lag phase, coupled with rapid growth of the two isolates, showed effective utilization of glyphosate by the selected isolates. *P. fluorescens* attained maximum growth and peaked at 132 h incubation, while *Acetobacter* sp. achieved maximum growth and peaked at 72 h incubation. There have been several reports on the ability of microorganisms, including some *Pseudomonas* sp., to effectively utilize glyphosate by naturally synthesizing appropriate enzymes or as a result of genetic mutation (Jacob et al., 1988; Shinabarger et al., 1984; Kishore and Jacob, 1987). So far, there has been no report on the ability of *P. fluorescens* to utilize glyphosate as sole phosphorus or carbon source. The high capacity of these two organisms to utilize this herbicide *in vitro* could be attributed to their previous contact with the herbicide in the soil (rice fields) from where they were isolated. It is also possible that the organisms have undergone genetic mutation leading to the adaptability of the organisms to their microenvironment.

The results of the comparative role of glyphosate as carbon or phosphorus source for the two isolates showed that glyphosate serves as a better phosphorous source than as a carbon source. Many bacterial isolates have been reported to utilize glyphosate as a phosphorus source (Liu et al., 1991; Balthazor and Hallas, 1986; Dick and Quinn, 1995). Of the two isolates used in the present study, *P. fluorescens* demonstrated a better capacity to utilize glyphosate as carbon and phosphorus source. Glyphosate served as a better phosphorus source than carbon source for the *Acetobacter* sp. The presence of inorganic phosphate in the growth medium affected the effective uptake of glyphosate in both organisms. This is because inorganic phosphate has been reported to sup-

press the genes coding for the C-P lyase system and thus make them unable to metabolise glyphosate (Liu et al., 1991). Our results in this study agree with this report.

The results of this study showed that increase in glyphosate concentration led to a concomitant decrease in the growth of the isolates. This is in contrast with the report of Amoros et al. (2007), who, while studying the effects of roundup (glyphosate) at different concentrations (50 and 100 mg/l) observed an increase in *Aeromonas* counts in contrast with the control which contained no glyphosate. However, Carlisle and Trevors (1988) observed that glyphosate can either stimulate or inhibit soil microorganisms depending on the soil type or herbicide concentration. In our study, high cell density was recorded for both isolates at glyphosate concentrations of 7.2 - 50 mg/ml after 24 h at 30°C. However, at higher concentrations (100 and 250 mg/ml), the cell density was very low compared with the control (7.2 mg/ml). Even though a severe decline in growth of the organisms occurred at high concentrations (100 and 250 mg/ml), the isolates were still able to tolerate 250 mg/ml of glyphosate. A possible explanation may be the presence of novel degradative systems in the organisms.

Conclusion

Herbicides are extensively used in farming practices. Some of the farmers, due to illiteracy and impatience, apply more than the stipulated quantity of herbicide per application. These chemicals may persist for long periods of time in the environment, whereby they may affect non-target organisms. This study reports the isolation and identification of two bacterial species, *P. fluorescens* and *Acetobacter* sp. that possess the capacity to utilize glyphosate. The ability of these isolates to utilize glyphosate effectively provides a means of removing this compound from the environment. Thus the capacity of the isolates to survive and grow in the presence of high concentrations of the herbicide marks them out as good candidates for the bioremediation of glyphosate-polluted environments.

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