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Isolation, Characterization and Biodegradation Assay of Glyphosate Utilizing Bacteria from Exposed Rice Farm

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Abstract
Two bacterial species capable of utilizing glyphosate were isolated from the long term glyphosate exposed rice farm and tested for their abilities to utilize glyphosate at different concentrations. The two bacterial isolates were identified by standard bacteriological methods as *Pseudomonas sp* and *Bacillus subtilis* using their morphological, biochemical and growth characteristics. The utilization of glyphosate by the isolates was studied by monitoring their biomass production in basal medium containing glyphosate as phosphorus source at 660nm wavelength using the spectrophotometer. The growth of the isolates in the medium with inorganic phosphate as phosphorus source and the effect of different concentrations of glyphosate on the growth and utilization of glyphosate were also studied by assessing the turbidity in the medium at 660nm wavelength. The isolates showed a significant growth in the basal medium containing glyphosate as phosphorus source ($P<0.05$). Nevertheless, the test organisms showed a better growth rate in the medium with inorganic phosphate as phosphorus source. The effect of different concentrations showed that *Pseudomonas sp* grew significantly at concentrations of 7.2 mg/ml to 40 mg/ml while *B. subtilis* showed a reduction in growth with corresponding increase in glyphosate concentration. The results of this study nonetheless underscored the efficacy of the test bacterial isolates in glyphosate utilization, hence their potential as candidates for bioremediation of glyphosate contaminated farmland in poor resource settings.
Keywords: Glyphosate herbicide, Utilization pattern, *Pseudomonas sp., Bacillus subtilis*.

INTRODUCTION

Glyphosate is a post-emergent and non-selective (or broad spectrum) herbicide used in both agricultural and non-agricultural areas [1]. Glyphosate is an organophosphate, containing carbon and phosphorus [2]. Glyphosate is a weak organic acid consisting of glycine and phosphonomethyl moiety. Its chemical name is N-(phosphonomethly) glycine. It is formulated as the Isopropyl amine or trimethylsulfonium salt of glyphosate [3]. Glyphosate is used to kill all plant types including grasses, perennial, and woody plants [4,5]. It can be used in non-till (zero tillage) agriculture to prepare field before planting [4].

Glyphosate is the active ingredient of the commonly used herbicide Roundup. Plants take the herbicides up very easily and then, it exhibits specific growth reduction by interfering upon the metabolism of essential aromatic compounds in the shikimic acid pathway [6]. It inhibits the enzyme 5-enolpyruval shikimate-3-phosphate (EPSP) synthase which is the major enzyme of shikimic acid pathway responsible for the biosynthesis of aromatic amino acids [7]. This pathway is present in higher plants and microorganisms but not in animals and therefore glyphosate appears to have no direct toxic effect on animals [3]. However, the toxicity effects on fishes and non target beneficial soil organisms have been reported [8,9].

Glyphosate is the world's bestselling herbicide [10]. This wide use can very much be ascribed to acclaimed high weed-killing efficiency, low toxicity to non-target organisms and apparently a very limited risk of leaching to groundwater because glyphosate seems to be inactivated in soils by strong sorption and relatively fast degradation [11,12].

The wide use, and hence ubiquity, of glyphosate makes great demands on glyphosate safety, i.e. the absence of any harmful environmental effect except on target organisms (the undesirable weeds). In spite of many papers [11,13,14], showing that glyphosate is relatively safe environmentally, recent investigations indicate possible leaching and toxicity problems with its use [15-17]. Therefore, it is necessary to find means of decontaminating the polluted environment by quickly removing or degrading the herbicide to reduce its effect on non-target organisms. The most effective way of removing glyphosate from the contaminated environment is by biological process as chemical process is ineffective due to the presence of direct C-P bond which is resistant to physicochemical effect [18].

Biological treatment employs substantial metabolic capacities of the microorganisms to transform organic pollutants into harmless or, at least, less dangerous compounds. In this method, reasonably, strains acclimatized to polluted site are used to enhance bioremediation process. Thus, several studies were initiated to isolate pure bacterial strains with degrading capability for potential uses in the biological treatment, such as removal of glyphosate from wastewater [19]. Nevertheless, many more ubiquitous bacterial strains with high nutritional versatility, enzyme production and high tolerance to environmental changes are still available for exploitation. Bacteria can utilize glyphosate as carbon source and leads to production of aminomethylphosphonic acid (AMPA) or as phosphorus source and produces glycine [20].

The aim of this work was to isolate Bacterial strain with potential ability to utilize glyphosate from the exposed rice farm as well as consider the changes in pH during the utilization and also the effect of different concentration of the herbicide on their utilization pattern.

MATERIALS AND METHODS

Collection of Soil Samples

Glyphosate contaminated soil was collected from two rice farms located in Agubia Ikwo in Ikwo LGA, Ebonyi State, Nigeria. Six soil samples were collected at three different points on each farmland at the depth of 0-10cm. The samples were transported immediately to the laboratory in a sterile polyethylene bags and analyzed within 24 h.

Enrichment Isolation

Isolation was done using a constituted basal medium with the following constituents g/l:- $MgSO_4$(EGA CHEMIE-Germany), 2.0; $CaCl_2$(M&B England), 1.0; NH_4Cl(M&B-England), 3.0; Glucose(BDH-England), 1% (w/v); Tris buffer(MERK-Germany), 6.05; Deionized water, 1000ml and filter sterilized glyphosate(Monsanto-USA) (7.2mg/ml) which is added after autoclaving the constituted media. This concentration of glyphosate (7.mg/ml) was used because it is equivalent to the site application concentration. The basal medium was prepared and dispensed into 250 ml Erlenmeyer flasks in duplicates. A total of 1.0g of each soil sample was added to separate Erlenmeyer flasks containing 50ml of the basal medium supplemented with 1.0ml of filter sterilized glyphosate (7.2mg/ml) as a sole phosphorus source. The cultures were incubated in an orbital shaker (Mk.V Orbital shaker-UK) at 30^0C and 120rpm for 7 days. An aliquot of 1.0ml was collected from the culture broth and transferred into freshly prepared basal medium and incubated for additional 7 days under the same conditions, after which 0.1ml aliquot was spread on the plates of basal agar medium containing glyphosate as sole phosphorus source and incubated at 30^0C for 5 days. Morphologically distinct colonies were isolated after several subcultures on the plates of basal agar medium. The ability of the isolates to utilize glyphosate was tested by inoculating a colony from each isolate into 250ml Erlenmeyer flasks containing 50ml of freshly prepared basal medium supplemented with 1.0ml of glyphosate (7.2 mg/ml) as sole phosphorus source. The flasks were incubated on a rotary shaker at 120 rpm for 216 h at 30°C. The ability of each isolate to utilize glyphosate was measured based on the turbidity of the medium at wavelength of 660 nm using a spectrophotometer. Two Baceterial species were selected for the study based on their outstanding glyphosate utilization pattern.

Characterization and Identification of Isolated Bacterial Strains

The identity of the isolates was affirmed after characterization by standard bacteriological methods as described by [21-23]. The identified isolates were kept in nutrient agar slant in the refrigerator till used.

Inoculums Preparation and Standardization

The inocula of the isolates used for this study were prepared by sub – culturing in nutrient broth and incubating for 24 h at 30^0C. The cultures were standardized by collecting aliquot and comparing the absorbance in spectrophotometer at wavelength of 600nm with that of MacFarad 0.5 nephrometer standard [7].

Time Course of Glyphosate Utilization by the Test Isolates

The Time Course of utilization of glyphosate by the two isolates (*Bacillus subtilis* and *Pseudomonas sp*) in basal medium broth containing glyphosate was monitored using spectrophotometer at 660 nm wavelength. Erlenmeyer flasks (250ml) each containing 50ml of basal medium of the following composition (g/l): $(NH_4)_2Cl$, 3.0; $MgSO_4$, 2.0; $CaCl_2$, 1.0; tris buffer 6.05; glucose, 1% (w/v), 1ml of filter sterilized glyphosate (7.2 mg/ml) as phosphorus source, 1000ml deionized water, pH 7.0 were inoculated in duplicates with each of the standardized cultures of the test isolates and incubated on a shaker incubator for 216 h at temperature of 30^0C and 120rpm. The utilization was monitored at 24 h interval by removing one set of Erlenmeyer flasks (i.e. two flasks) containing the culture to assay for biomass production using spectrophotometer at 660nm wavelength. A medium without organism was also prepared as control.

Growth Pattern of the Test Isolates in a Phosphate Minimal Medium

The Growth pattern of the test isolates in a phosphate minimal medium that contains inorganic phosphate as a sole phosphorus source was studied in a shake flask cultures. Erlenmeyer flasks (250ml) each containing 50ml of basal medium of the following composition (g/l): $(NH_4)_2Cl$, 3.0; $MgSO_4$, 2.0; $CaCl_2$, 1.0; K_2HPO_4, 0.5; KH_2PO_4, 0.5; glucose, 1% (w/v), 1000ml deionized water, pH 7.0 were prepared in duplicates and inoculated with each of the standardized cultures of the test isolates and incubated at temperature 30^0C and 120rpm. The utilization of the medium was monitored at 24 h interval by removing one set of Erlenmeyer flasks (i.e. two flasks) containing the culture to assay for biomass production using spectrophotometer at wavelength of 660nm.

Effect of Different Concentrations of Glyphosate on the Utilization Pattern by the Test Isolates

Aliquots (1.0 ml) of the standardized culture of the test organisms were inoculated into 250ml Erlenmeyer flasks containing 50ml of basal medium supplemented with various concentrations of glyphosate mg/ml: 20, 40, 60, 100, 150 and 200. A control was maintained with basal medium supplemented with 7.2 mg/ml of glyphosate. All the preparations were done in duplicates. The inoculated cultures were incubated for 120 h on a rotary shaker at 120rpm and temperature of 30^0C. Bacterial growth was monitored by removing one set of flask for each concentration of glyphosate at 24 h interval starting from 0 h which was taken immediately after inoculation to measure the optical density of the medium at the wavelength of 660nm.

Statistical Analysis

The results obtained at different stages of this study were analyzed statistically using analysis of varience (ANOVA) and Independent *T*-test using SPSS statistical package, version 16.0. Separation of means for statistical significance was done using LSD at 5% probability level [24].

RESULTS

Characterization and Identification of Isolated Bacterial Strains

Two pure cultures of bacterial species designated as isolates A and B which showed outstanding growth pattern in minimal media containing glyphosate as a source of phosphorus were generated through enrichment procedure. The result of characterization of the isolates showed that isolate A was a short Gram Negative, motile rod while isolate B was a gram positive, motile and spore positive rod. Further Biochemical, Morphological and Growth characterizations confirmed isolate A as *Pseudomonas sp.* and isolate B as *Bacillus subtilis*.

Time Course of Glyphosate Utilization by the Test Isolates

The time course of glyphosate utilization by the test isolates (*Bacillus subtilis* and *Pseudomonas sp.*) is shown in Figure 1 and 2 respectively. There was significant growth ($P<0.05$) of the isolates with corresponding decline in the culture pH. The growth of *Bacillus subtilis* peaked at 48 h corresponding to the pH of 5.75 while that of *Pseudomonas sp* peaked at 96 h and pH 6.15. Glyphosate utilization by the two organisms is compared in Figure 3. The result showed that *Bacillus subtilis* grew faster than *Pseudomonas sp* and but the growth peaked earlier.

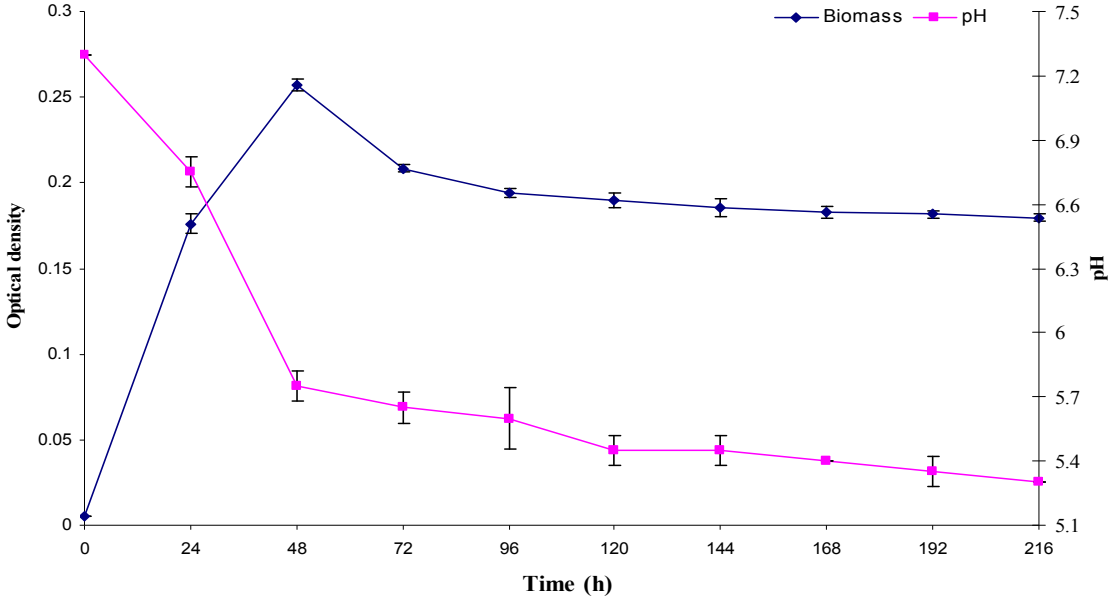

Figure 1: Growth curve of *Bacillus subtilis* showing utilization of glyphosate as phosphorus source and pH changes in the medium

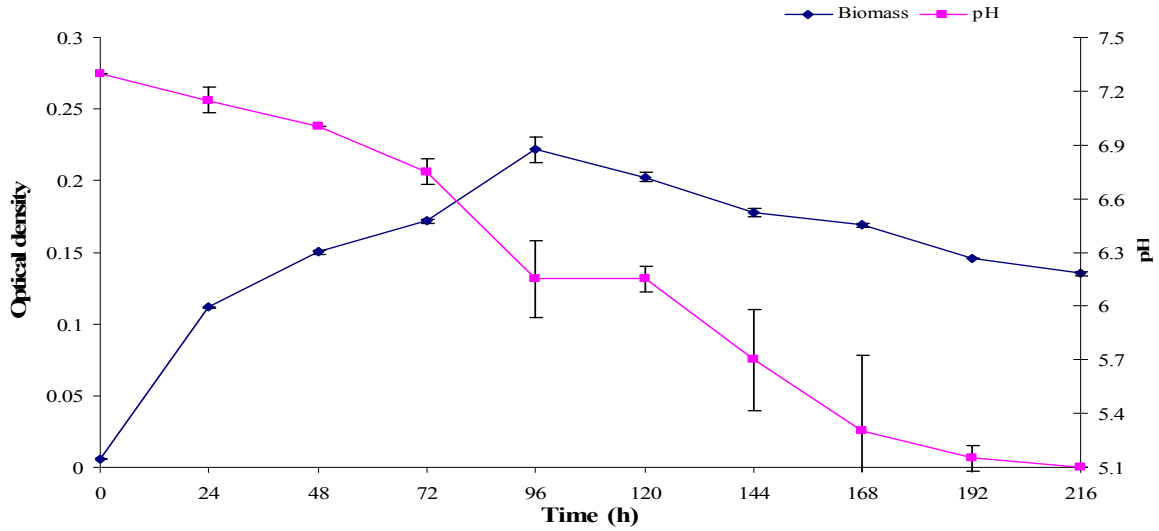

Figure 2: **Growth curve of *Pseudomonas sp* showing utilization of glyphosate as phosphorus source and pH changes in the medium**

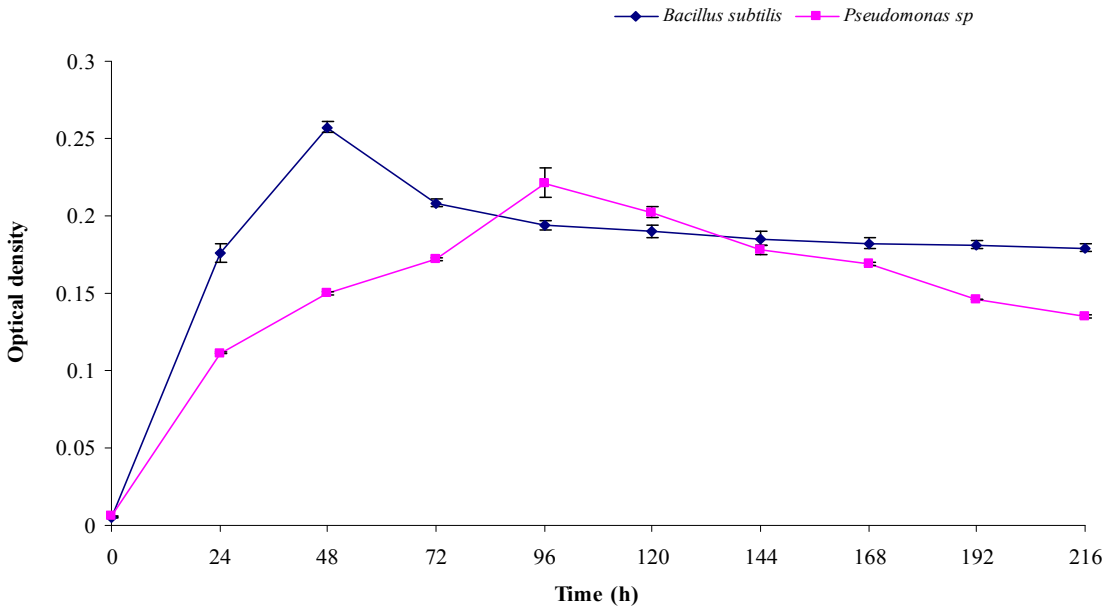

Figure 3: **Growth curve of *Bacillus subtilis* and *Pseudomonas sp* showing utilization of glyphosate as phosphorus source**

Growth Pattern of the Test Isolates in a Phosphate Minimal Medium

The growth pattern of in a complete medium with inorganic phosphate as sole source of phosphorus and pH changes in the medium is presented in Figure 4. Efficient utilization of the medium by *Bacillus subtilis* was apparent. A very sharp increase in growth rate with correspondingly sharp decline in pH of the medium was observed following 24 h incubation. In spite of the normal microbial growth curve, however, there was no apparent lag phase: a peak in the growth occurred at 72 h of incubation, followed by a long stationary phase, a decline phase after 192 h of incubation at a pH of 3.7.

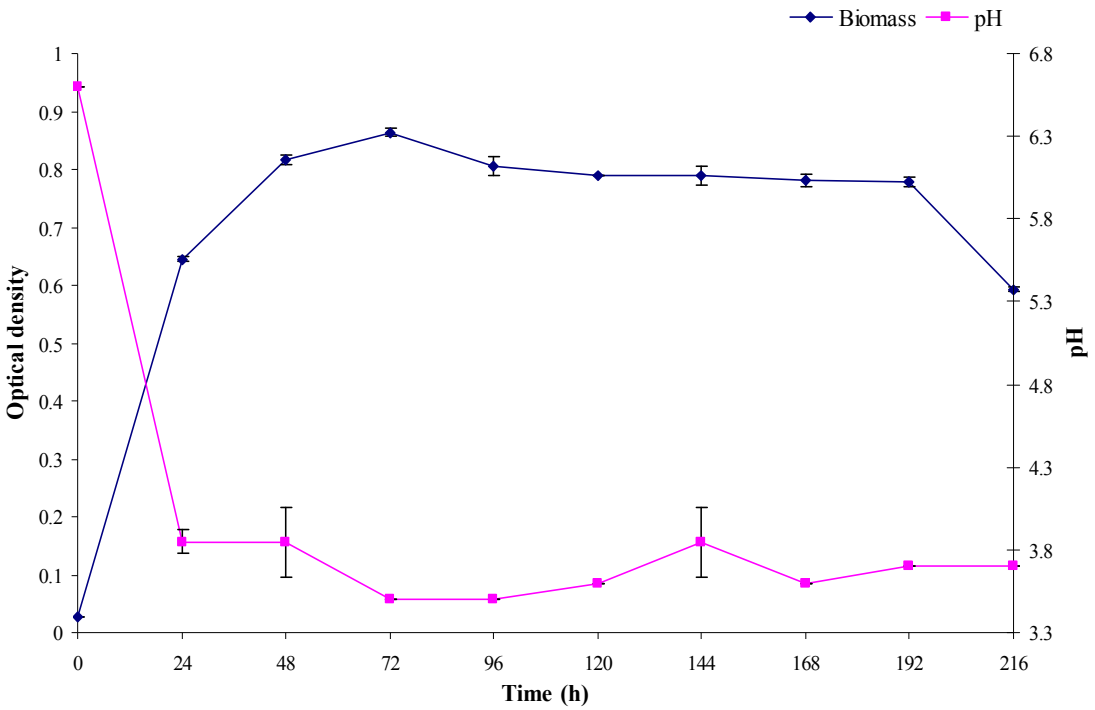

Figure 4 : Growth curve of *Bacillus subtilis* showing utilization of inorganic phosphate as phosphorus source and pH changes in the medium

The paradigm of *Pseudomonas sp.* growth in a complete medium presented in Figure 5 showed a normal growth curve with a peak after 120 h incubation at pH of 3.3. However, a short stationary phase was observed before a subsequent decline after 144 h of incubation at pH of 3.3.

Effect of Different Concentrations of Glyphosate on the Growth of the Test Isolates

The growth profile of *B. subtilis* in the medium with different concentrations of glyphposate was inversely proportional to the glyphosate concentration (Figure 6). As the glyphosate concentration increased, there was a corresponding decrease in the growth rate. The highest growth was observed in the control (7.2 mg/ml) which contained the least concentration of glyphosate followed by the growth in 20 mg/ml. There was no significant growth in other concentrations used.

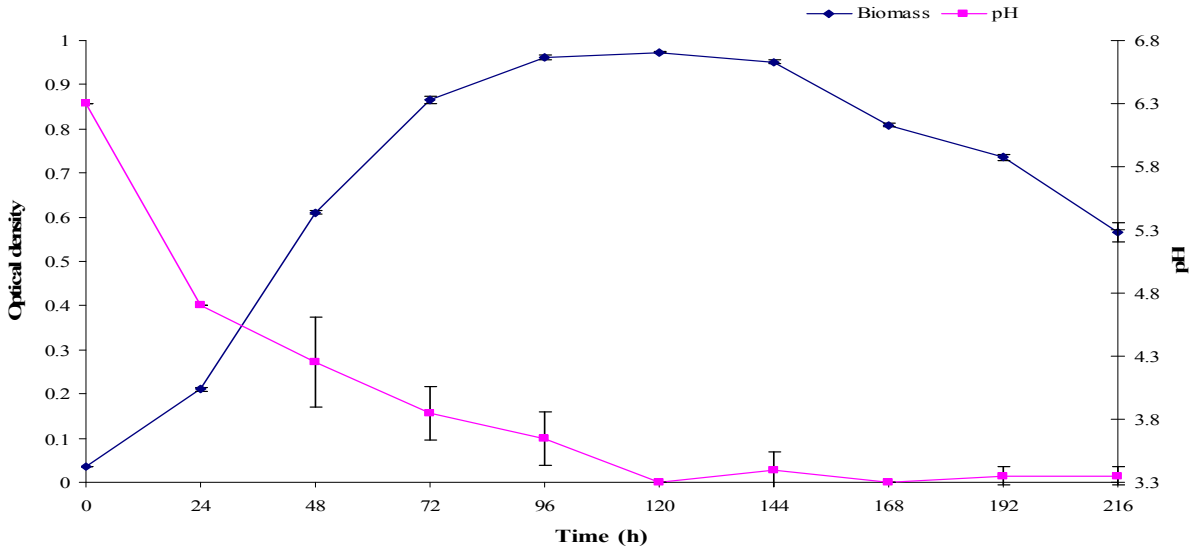

Figure 5: Growth curve of *Pseudomonas sp* showing utilization of inorganic phosphate as phosphorus source and pH changes in the medium

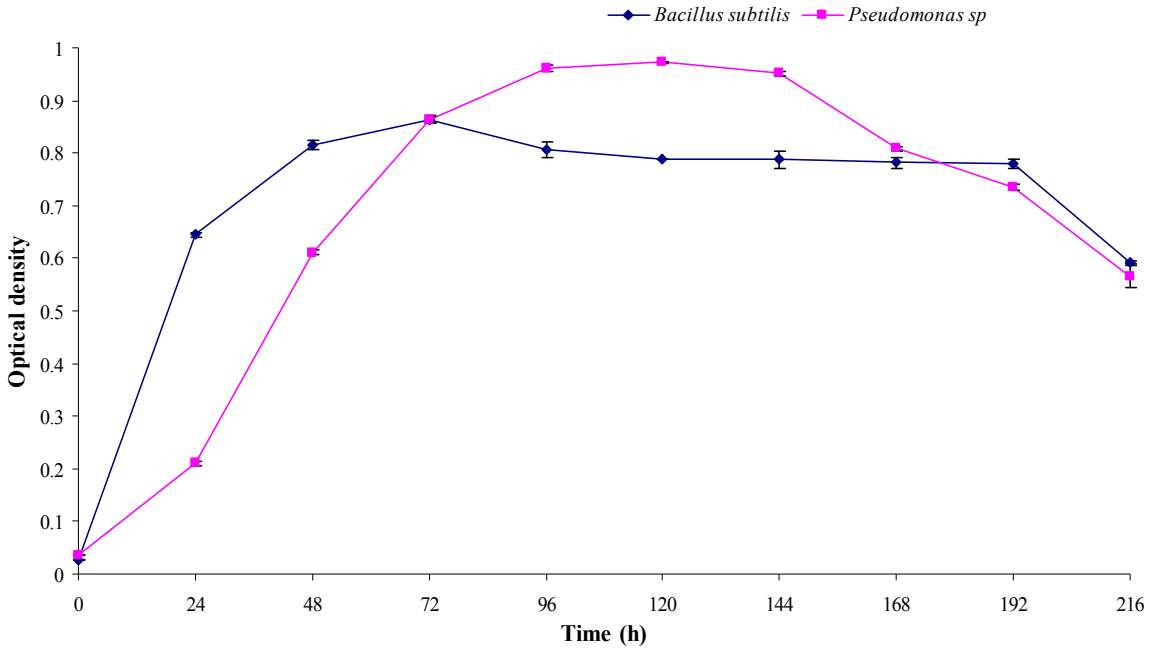

Figure 6: **Growth curve of *Bacillus subtilis* and *Pseudomonas sp* showing utilization of inorganic phosphate as phosphorus source**

On the other hand, *Pseudomonas sp.* exhibited a different pattern of growth in this medium; a significant growth was observed over a wide range of concentrations (Figure 7). Growth profile in the medium with glyphosate concentrations of 20 mg/ml and 40 mg/ml was better compared with the growth profile in the control medium (i.e 7.2 mg/ml) which had the least concentration of glyphosate. An interesting observation made in all the concentration tested was that *Pseudomonas sp* exhibited a brief lag growth till the end of 24 h of incubation except in the control (7.2 mg/ml) in which growth started increasing at 24 h of incubation but peaked at 48 h and continued with a steady or constant rate till the end of monitoring. Highest growth rate in the medium with glyphosate concentration of 40 mg/ml was thus observed, peaking at 120 h of incubation followed by the growth in the medium with the concentration of 20 mg/ml with a peak at 48 h of incubation. Growth in other concentrations tested showed a reverse pattern, decreasing with the increase in glyphosate concentration. Nevertheless, Statistical analysis of the result showed no significant difference ($P<0.05$) between the growth in the 20 mg/ml and 40 mg/ml concentrations. However, a significant difference ($P>0.05$) was observed between the two concentrations (20 mg/ml and 40 mg/ml) and other concentrations tested.

The comparison of the growth rate of the two organisms tested (*Bacillus subtilis* and *Pseudomonas sp*) in different concentrations of glyphosate is shown in Figures 8 and 9. *Pseudomonas sp* exhibited a higher growth pattern in all the concentrations tested than *B. subtilis.* However, the growth of *B. subtilis* was higher in the control concentration (7.2 mg/ml). There was nonetheless significant difference ($P<0.05$) in the growth of the two organisms in the concentration of 20 mg/ml and 40 mg/ml but no significant difference in other concentrations tested.

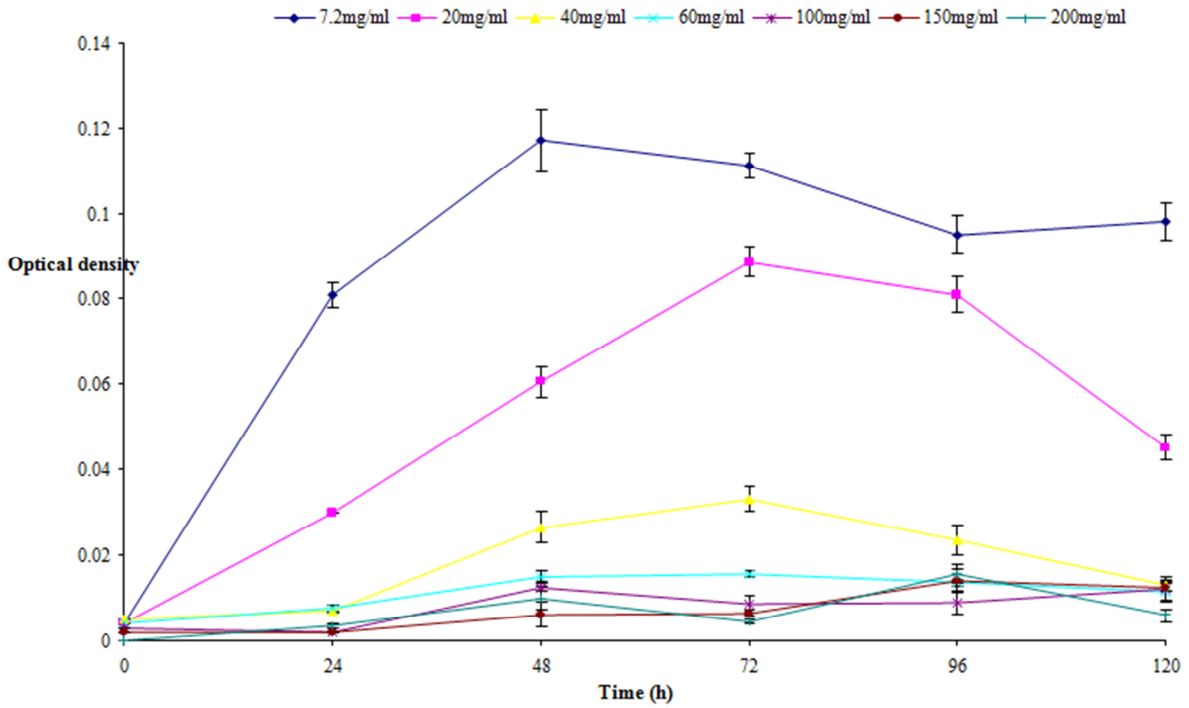

Figure 7: Growth kinetic of *Bacillus subtilis* on different concentrations of glyphosate as phosphorus source

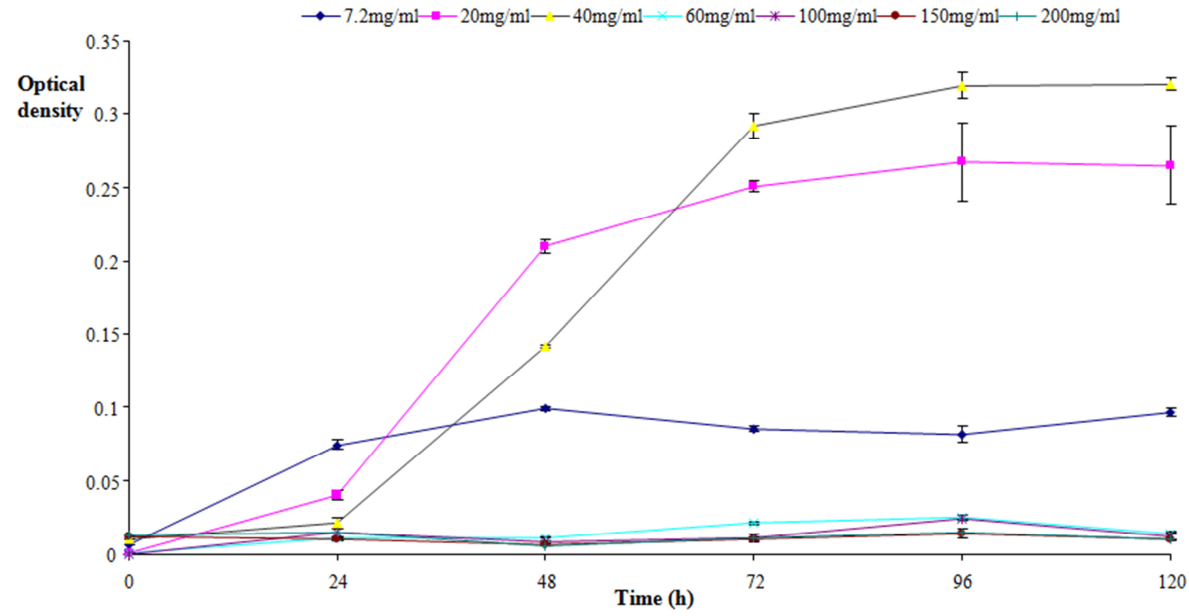

Figure 8: Growth kinetic of *Pseudomonas sp* on different concentration of glyphosate as phosphorus source

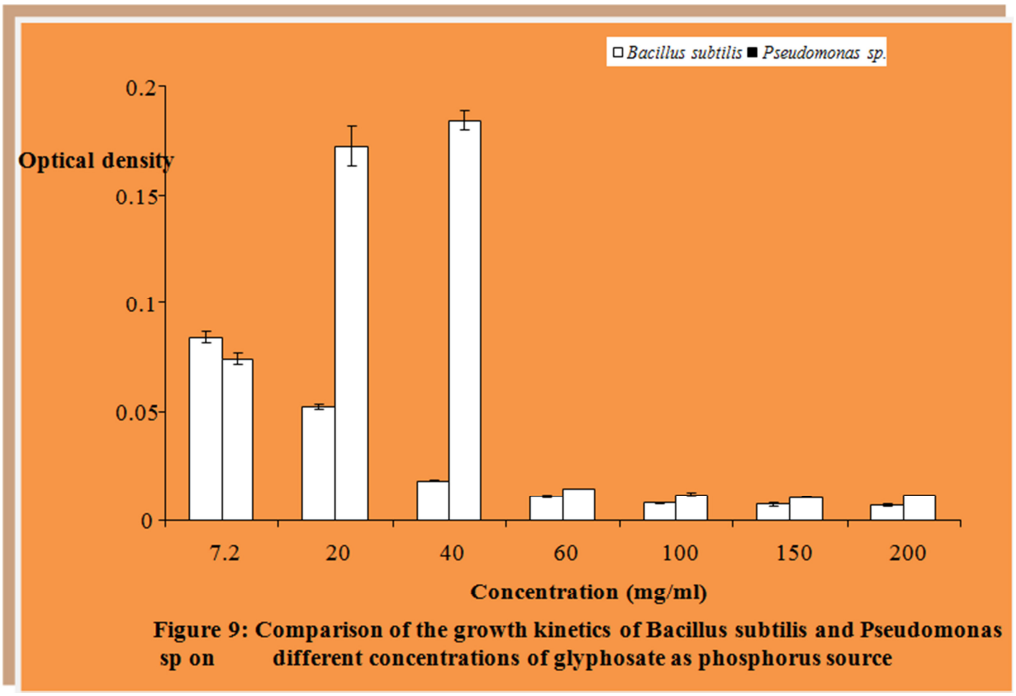

Figure 9: Comparison of the growth kinetics of Bacillus subtilis and Pseudomonas sp on different concentrations of glyphosate as phosphorus source

DISCUSSION

Bacterial strains with novel capabilities in glyphosate utilization were isolated and investigated for their glyphosate utilization capabilities. These organisms showed a high potential in utilizing glyphosate as observed by their significant growth in the presence of glyphosate as a sole phosphorus source. Though many researchers have reported the isolation of glyphosate utilizing *Pseudomonas sp.* [25-27], nonetheless, none has, to the best of our knowledge reported isolation of *Bacillus sutilis* capable of utilizing glyphosate. In the present study, the *Bacillus subtilis* isolated did not just only utilize glyphosate but also compares favourably with *Pseudomonas sp* in glyphosate utilization, implying that there are still many potential organisms that can be used for cleaning up glyphosate contaminated environment which are yet to be exploited. However, the high competence shown by the isolated organisms; *B. subtilis* and *Pseudomonas sp*. in this study could be attributed to their source which is a site subjected to prolonged contamination by glyphosate implying that the organisms might have undergone gene mutation and become adapted to the glyphosate and therefore could utilize the chemical for their growth. On the other hand, their potential for glyphosate utilization could be due to their ability to elaborate enzyme for glyphosate utilization following exposure to the herbicide. This opinion agrees with several reports on the ability of microorganisms including some species of *Pseudomonas* to effectively degrade glyphosate by naturally synthesizing appropriate enzymes or as a result of genetic mutation [25,28].

The decline in pH as observed in the result could be attributed to the utilization pathway adopted by the test organism. Microorganisms have been observed to be able to degrade glyphosate through two major pathways namely: the sarcosine pathway and aminomethylphosphonic acid (AMPA) pathway [28,10]. AMPA pathway leads to the production of AMPA intermediate which can reduce the pH of the medium as evidenced by the result of this work, which showed that the pH of the medium dropped simultaneously with the utilization of glyphosate in the medium. On the other hand, if the sarcosine pathway which involves direct cleavage of C-P bond by C-P lyase was followed, then some other product could have been responsible for the decline in pH of the medium.

The results of inorganic phosphate utilization as phosphorus source showed that the test organisms thrived well in this medium. This result is in agreement with the report of [25] which indicated that microorganisms can use glyphosate as either carbon, phosphorus or nitrogen source, though they grow better on inorganic sources of C, P or N. This observation could be attributed to the inhibitory activity of glyphosate to these organisms, or due to the stress involved in elaboration of enzyme required in the degradation of glyphosate for their uptake therefore the reason why the organisms grew better in this medium containing these nutrients in their elemental form since they can easily access them for their growth. The observed decline in pH of this medium showed that the fall in pH observed in glyphosate minimal medium discussed earlier could have been due to other metabolic products of these organisms other than aminomethylphosphonic acid (AMPA), implicating the use of sarcosine pathway in glyphosate utilization which involves the cleavage of C-P bond to release phosphorus instead of the aminomethylphosphonic acid (AMPA) pathway, involving the cleavage of C-

N bond to release AMPA as earlier reported [28,10].

Degradation of glyphosate therefore seems to be a cometabolic process, because some microorganisms are not able to use glyphosate as a carbon source [10,29]. Available results of this study therefore underscore the potential of the test organisms to utilize glyphosate as phosphorus source but not as carbon source. This now is in consonance with other reports which indicated that phosphate starvation leads to the uptake of glyphosate as source of phosphorus which involves the cleavage of C-P bond, one of the major pathways of glyphosate degradation catalyzed by C-P lyase. [30,31]. The C-P lyase activity is induced under phosphate starvation and microorganisms can therefore use phosphonate as alternative phosphorus sources as reported by [31]. These observations give credence to the reported potential of our test organisms to utilize glyphosate in the medium with glyphposate as sole phosphorus source since they were being starved of inorganic phosphate.

The result of the effect of different concentrations of glyphosate on glyphosate utilization is remarkable. It was observed that when the concentration was increased from the control (7.2 mg/ml which is the site application concentration, to higher concentrations of 20 mg/ml and 40 mg/ml), there was a sharp increase in the growth of the *Pseudomonas sp*. This report is in consonance with the report of [32] in which a significant increase in growth ($P<0.05$) was observed in *Aeromonas sp.* following increase in the concentration of glyphosate up to 50 and 100mg/L respectively. In another similar study, 5 *Pseudomonas* species were not inhibited by glyphosate, but a 6^{th} species, *Pseduomonas fluorescens*, was inhibited [37]. However, the current study disagrees with the finding of [7] which reported that the growth of the organisms is inversely proportional to the glyphosate concentration, implying that as the glyphosate concentration increases, the growth of the organism subsequently decreases accordingly. Conversely, higher concentrations of glyphosate up to 60 mg/ml and above had a very serious effect on the growth of the organisms as reported by our study. Nonetheless, the pattern of growth exhibited by our test organism, *B. subtilis* was observed to be similar to what was reported by [7] in which microbial growth on glyphosate medium was inversely proportional to the concentration of glyphosate in the medium, implying that the higher the concentration, the lower the growth rate but is in disagreement with the finding of [32] which observed stimulation of growth of *Aeromonas sp* following increase in the concentration of glypghosate in the environment. This could be attributed to the degradation capabilities of the test organism *B.subtilis* and the level of enzyme activities it can elaborate for glyphosate degradation. However, the organism was very effective at the site application concentration (7.2mg/ml).

Though the test organisms still tolerated glyphosate up to 200 mg/ml used, however, the growth observed from 60 mg/ml and above was not very significant and this could be explained by the fact that at higher concentrations, pollutants or chemicals can become toxic to the microorganisms that can degrade them. This study therefore discloses the fact that at higher concentration of glyphosate, bio-augmentation may be needed to cushion the effect. The study further indicates the outstanding potential of the isolate *Pseudomonas sp.* to degrade glyphosate at wide ranges of concentrations than *B. subtilis*, implying the efficacy of *Pseudomonas sp* as an effective organism in the bioremediation of highly contaminated sites. This view is in consonance with the report of many researchers who put *Pseudomonas sp.* in the fore-front in the degradation of glyphosate [25,27,10].

CONCLUSION

Glyphosate usage is increasing daily due to some beneficial effect such as the broad spectrum and short term activities of the herbicide not minding the environmental and health impact. Owing to this observed beneficial effect, people tend to use it indiscriminately, leading to abuse. Glyphosate have been found to persist in the soil affecting non target organisms including mychorrhizal fungi and nitrogen fixing bacteria. This study reported the isolation of bacterial species (*Pseudomonas sp* and *Bacillus subtilis*) capable of utilize glyphosate effectively even at high concentrations especially *Pseudomonas* sp.

The result obtained from this study also revealed that glyphosate like some other xenobiotics can be toxic to organism that can degrade it when present in higher concentrations and therefore noted that though glyphosate may be degraded by indigenous soil organisms following application. However, in the event of heavy contaminations or prolonged exposure of a particular environment, bio-augmentations may be needed to cushion the effect. Therefore, the ability of our isolates especially *Pseudomonas sp.* to still withstand high concentrations of the herbicide makes them potential candidates for this purpose.

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ISOLATION OF GLYPHOSATE UTILIZING BACTERIA FROM EXPOSED RICE FARM AND THE EFFECT OF DIFFERENT CONCENTRATIONS ON THE UTILIZATION PATTERN

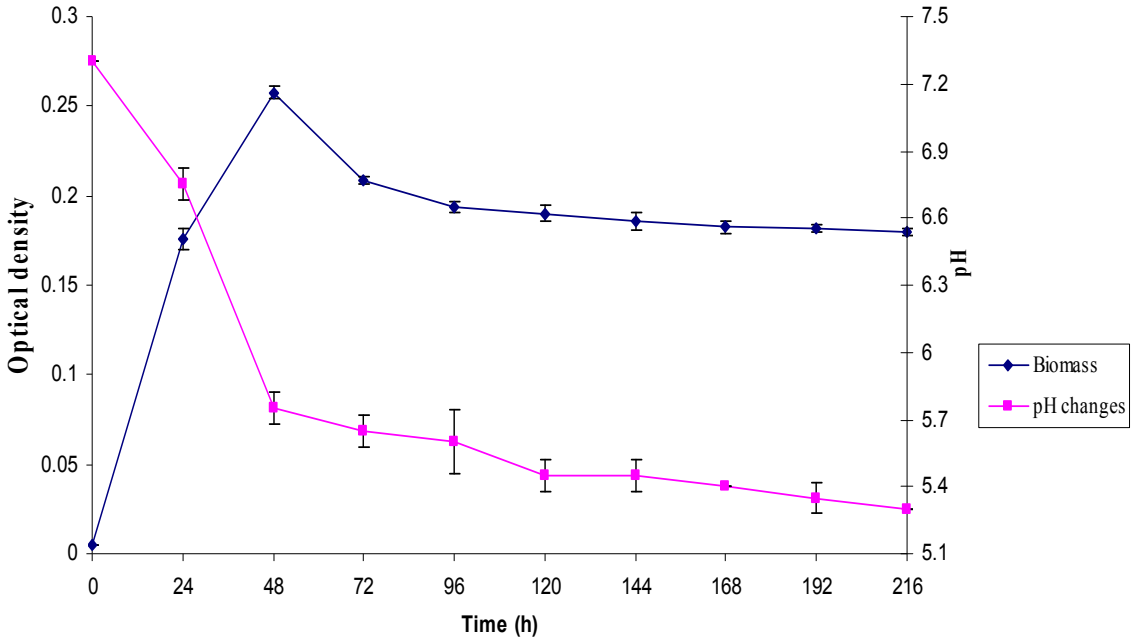

Figure 1: Growth curve of *Bacillus subtilis* showing utilization of glyphosate as phosphorus source and pH changes in the medium

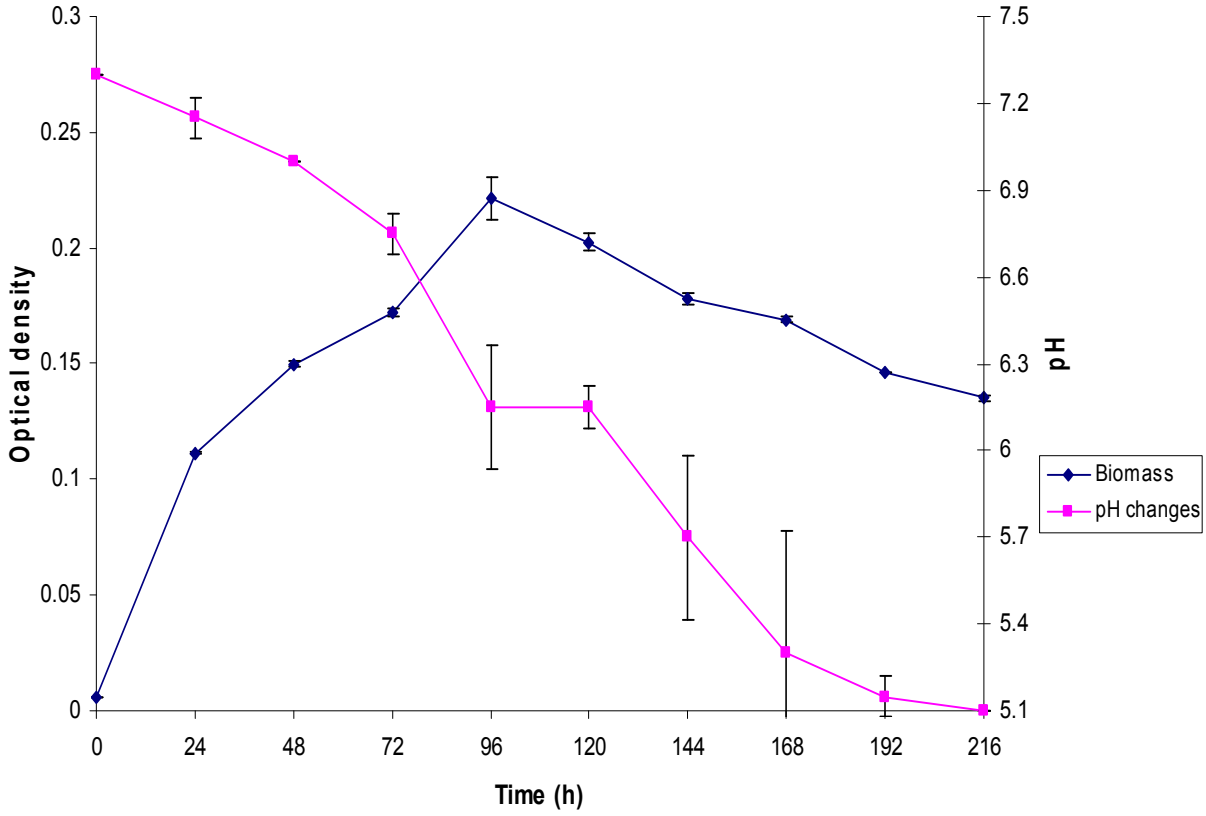

Figure 2: Growth curve of *Pseudomonas sp.* showing utilization of glyphosate as phosphorus source and pH changes in the medium.

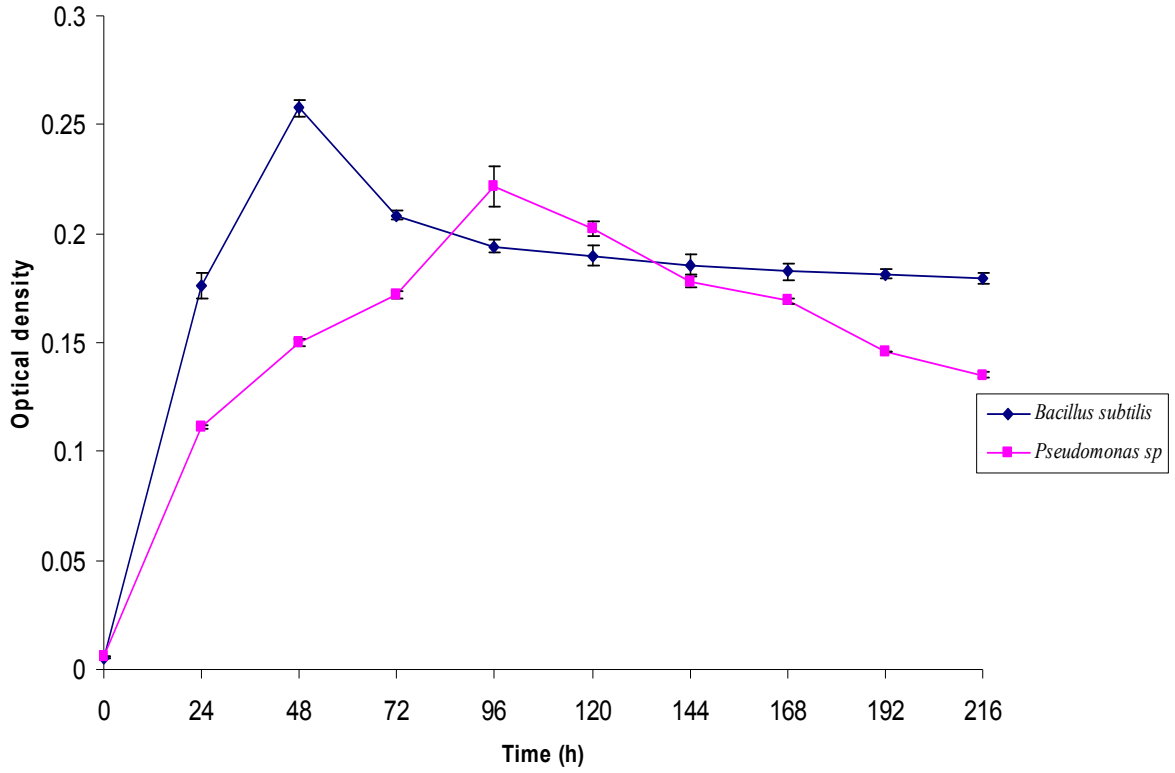

Figure 3: Growth curve of *Bacillus subtilis* and *Pseudomonas sp* . showing utilization of glyphosate as phosphorus source

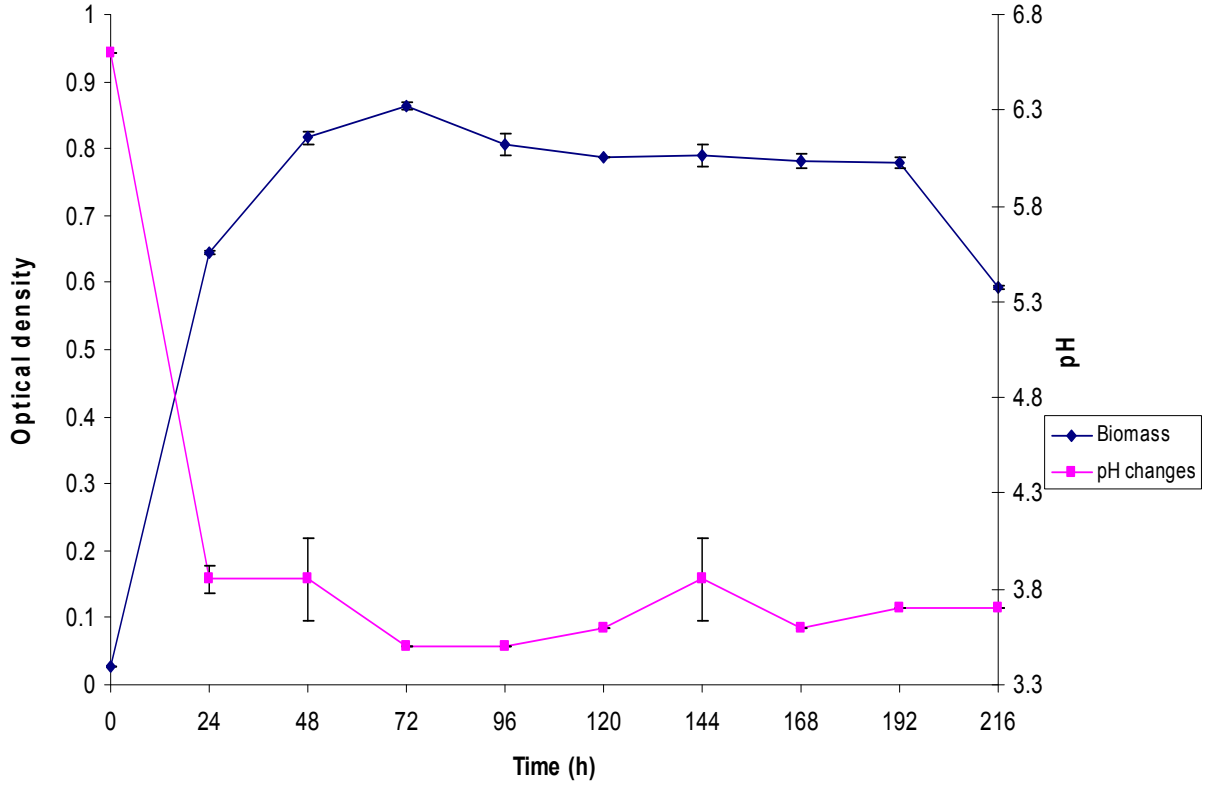

Figure 4: Growth curve of *Bacillus subtilis* showing utilization of inorganic phosphate as source of phosphorus and pH changes in the medium.

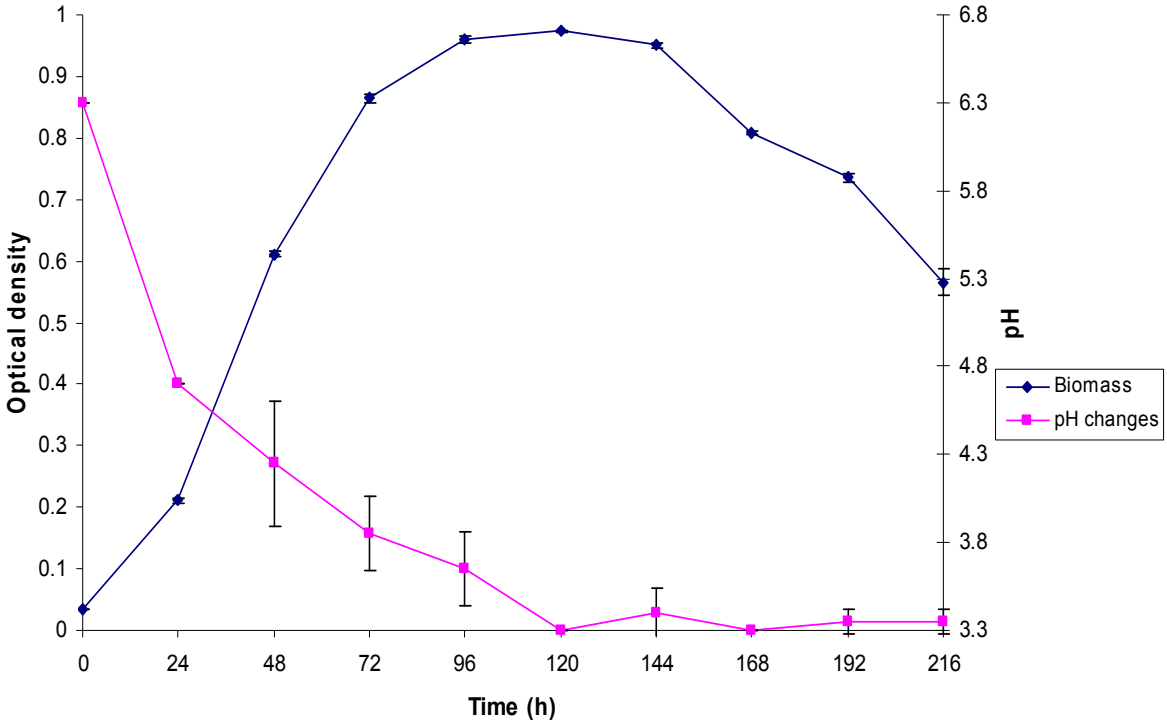

Figure 5: Growth curve of *Pseudomonas sp*. showing utilization of inorganic phosphate as phosphorus source and pH changes in the medium.

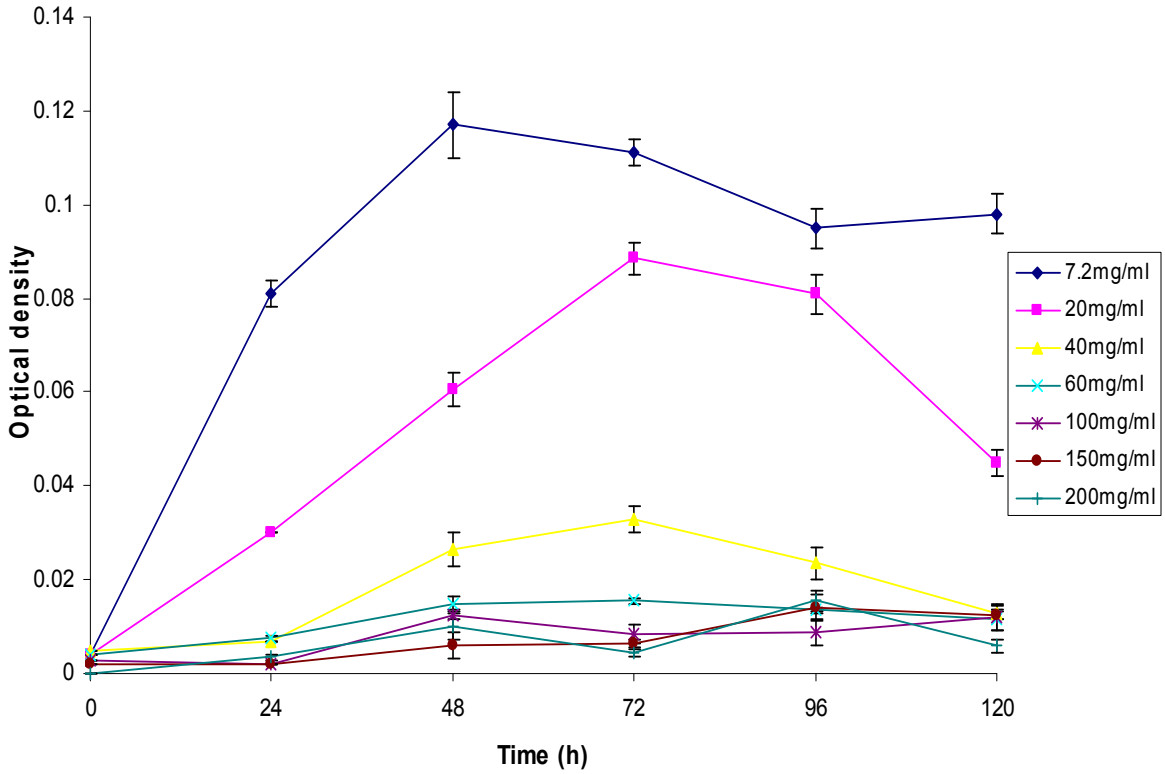

Figure 6: Growth kinetic of *Bacillus subtilis* on different concentrations of glyphosate as phosphorus source.

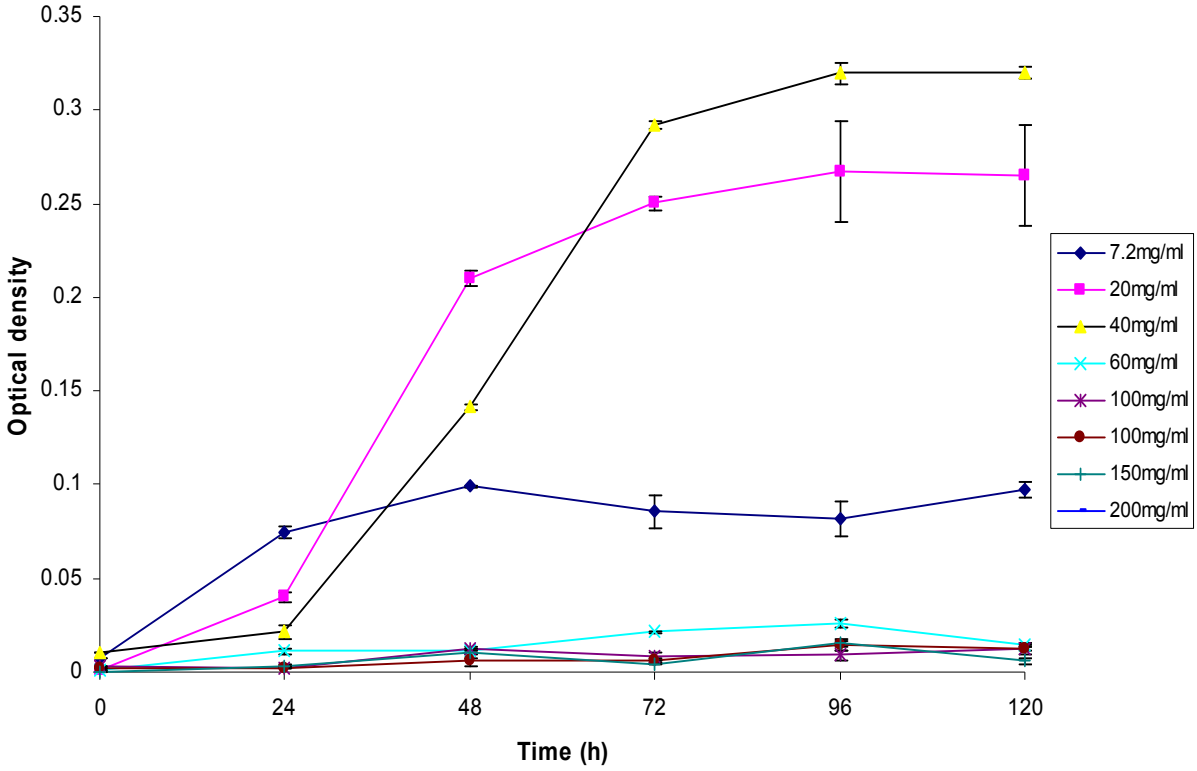

Figure 7: Growth kinetic of *Pseudomonas sp*. on different concentrations of glyphosate as phosphorus source.

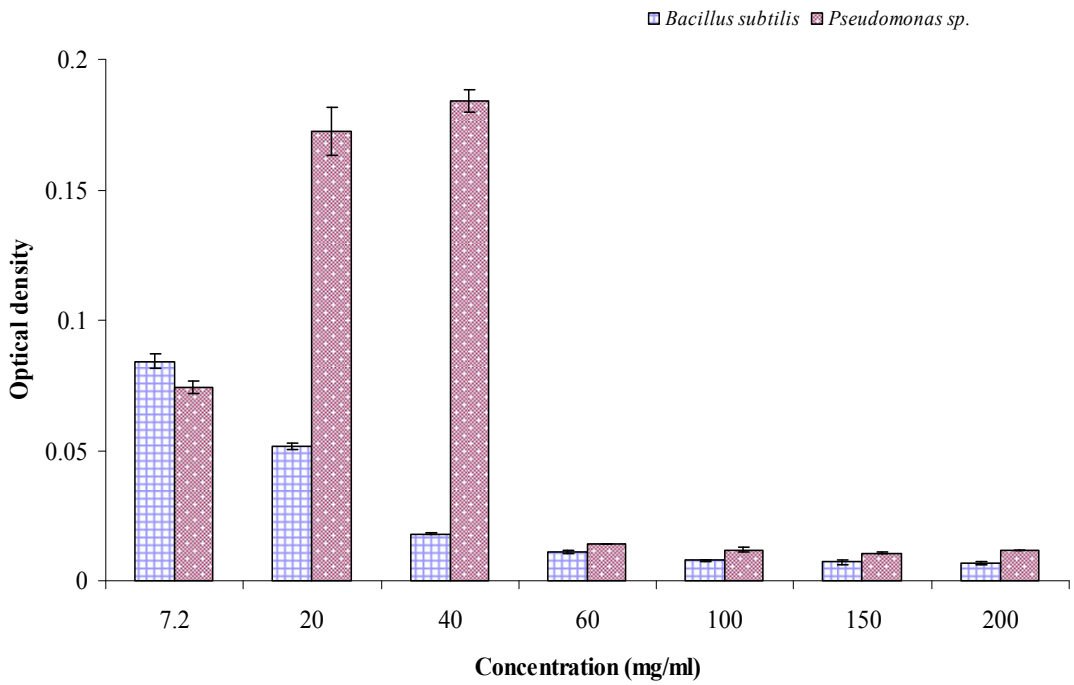

Figure 8: Comparison of the growth kinetics of Bacillus subtilis and Pseudomonas sp on different concentrations of glyphosate as phosphorus source

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