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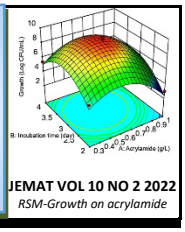

Glyphosate Biodegradation by Molybdenum-Reducing *Pseudomonas* sp.

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ABSTRACT

Bioremediation of pollutants, such as herbicides, is an economic and environmentally friendly process. Glyphosate is an active ingredient in most herbicides utilized for weed control and desiccation on cereal and other grain crops globally. Glyphosate pollution poses a threat to the environment and the habitats in it. In this study, an isolated molybdenum-reducing bacterium was characterized for its potential to degrade glyphosate and utilized as the sole source of carbon and electron donor. The effects of incubation time, glyphosate concentration (carbon source), inoculum size, pH, temperature, aeration and heavy metals on the growth of this bacterium were spectrophotometrically assayed as OD_{600} nm. The bacterium degrades glyphosate faster under shaking conditions, optimally at pH 7.0, concentration 1.0 g/L, temperature 40 ºC, and inoculum size 400 µL. Growth of this bacterium was significantly inhibited by heavy metals in the order of Cu>Zn>Pb>Hg>Ag>Fe compared to the control. Glyphosate can serve as an electron donor source in hexavalent molybdenum reduction, but poorly supports molybdenum blue (Mo-blue) production compared to glucose. The dual role of this isolate as a metal reducer and glyphosate degrader makes it unique already and an important instrument for the bioremediation of mixed pollutants.

INTRODUCTION

Environmental pollution is reaching worrying proportions worldwide. Urbanization and industrialization along with economic development have led to an increase in energy consumption and waste discharges. Global environmental pollution, including greenhouse gas emissions and acid deposition, as well as water pollution and waste management, is considered an international public health problem, which should be investigated from multiple perspectives including social, economic, legislative, and environmental engineering systems, as well as lifestyle habits helping health promotion and strengthening environmental systems to resist contamination [1]. Environmental pollutants have various adverse health effects from early life, some of the most important harmful effects are perinatal disorders, infant mortality, respiratory disorders, allergy, malignancies, cardiovascular disorders, increase in stress oxidative, endothelial dysfunction, mental disorders, and various other harmful effects [2]. Numerous studies have exposed that environmental particulate exposure has been linked to increased risk of morbidity and mortality from many diseases, organ disturbances, cancers, and other chronic diseases [3,4].

Glyphosate is a non-selective, systemic herbicide that can control most annual and perennial plants. Glyphosate residues in these products are causing public health concerns regarding their exposure [5]. Animal trials found that high doses of glyphosate damaged the organs, reproduction and nerve systems; however, limited human evidence supports the carcinogenicity of glyphosate exposure in humans. Due to the primary application of glyphosate in agriculture, glyphosate has been recovered from environmental samples such as water [5]. However, because of the growing evidence of glyphosate toxicity for living organisms, the problem of preventing its accumulation in natural

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environments (both soils and water bodies) and of its subsequent removal is becoming a topical issue. The only appropriate solution seems to be the use of microorganisms capable of degrading phosphonate xenobiotics into biologically safe compounds [6].

Globally, it has been estimated that about 125,000 – 130,000 metric tons of pesticides are applied every year [7]. Glyphosate usage has been banned in over 18 different countries all over the world [8]. In Nigeria, millions of farmers are still using this chemical without using protective measures [8]. Due to the concern for its toxicity, glyphosate bioremediation has been suggested as an alternative to the physicochemical methods of glyphosate degradation. Literature on the microbial ability to degrade and utilize glyphosate is vast, however, information about the use of glyphosate as an electron donor source to reduce any metal is lacking. [9] were first to report the utilization of glyphosate as a source of phosphate not [H+] for molybdenum reduction. This work will therefore focus on characterizing the potential of previously isolated molybdenum-reducing bacteria for glyphosate degradation and its possible utilization as an electron donor for molybdenum reduction. is a non-selective, systemic herbicide that can control most annual and perennial plants.

MATERIALS AND METHODS

Chemicals

All chemicals used in this work were of analytical grade. Glyphosate Forceup 750 SG was obtained from Zhejiang Jinfada Biochem. Co. Ltd., Hengcun Town, Tonglu Country, Zhejiang Province, China. Media preparation was based on the recipe [9] except otherwise stated here. All the experiments involving microorganisms were done in a class II biosafety cabinet.

Screening of isolate

A total of seven (7) previously isolated molybdenum-reducing bacteria were screened for their ability to degrade and utilize glyphosate on mineral salt agar media with the following composition in g/L; 0.5 g of NaCl, 0.5 g of KCl, 2 g of NH_4SO_4, 0.2 g of $MgSO_4.7H_2O$, 0.01 g of $CaCl_2$, 0.001 g of $FeSO_4$, 0.6 g of Na_2HPO_4, 1.5 g of KH_4PO_4, 18 g of agar (solidifying agent) and 1 g of glyphosate as sole carbon source. The pH of the medium was adjusted to 7.5, then autoclaved at 121 ºC, 115 kPa for 15 min, and glyphosate was autoclaved separately and added to the medium upon cooling afterwards. The medium was poured into a sterile disposable petri dish and allowed to solidify. Bacterial inoculum (100 µL) from a freshly prepared nutrient broth was dispensed onto the dishes and incubated for 24 h at 37 °C. Bacterial growth was observed using a "Colony counter". *Pseudomonas* sp. showed remarkably higher and faster growth within 48 h.

Characterization of glyphosate degradation

The factors (pH, concentration, temperature, heavy metals, inoculum size and aeration) that affect the growth of the bacterial isolate during glyphosate degradation were characterized based on one factor at a time (OFAT). Replicate experiments were carried out in Vijou bottles containing 10 mL sterile MS medium inoculated with 100 µL of the bacterial isolate [9].

Effect of incubation time

The effect of time on bacterial growth was observed at 0 h, 24 h, 48 h, 54 h and 72 h. 10 mL of MSM was dispensed into the Vijou bottles and inoculated with 100 µL bacterial suspension. Optical density (OD) at 600 nm was measured at 0 h, 24 h, 48 h, 54 h and 72 h to determine bacterial growth.

Effect of glyphosate concentration (carbon source)

Since glyphosate was utilized as the carbon source, the concentration of the pollutant was increased, varying the range from 0.5 – 10 g/L (0.5 g/L, 1 g/L, 2 g/L, 4 g/L, 8g/L and 10 g/L). The procedure was described above.

Effect of inoculum size

The inoculum size ranging from 50 – 1000 µL (50 µL, 100 µL, 200 µL, 400 µL, 600 µL and 1000 µL) was used to determine the optimum inoculum needed for bacterial growth. The procedure was as described above.

Effect of initial pH

The pH of the medium was set from 5.5 – 8.5 (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5) to determine the optimum pH for the growth of the bacterium.1 M NaOH and 50% HCl were used to adjust to various pH. The procedure was as described above.

Effect of temperature

The temperature was ranging from 30 – 45 ºC (30 ºC, 35 ºC, 40 ºC and 45 ºC) to determine the best temperature that supports bacterial growth and glyphosate degradation. The procedure was as described above.

Effect of aeration (shaking)

The effect of shaking and static incubation was observed at various time intervals (0 h, 24 h and 48 h) by placing the culture media on a shaker at 120 rpm, while the control experiment was kept static. The procedure was as described above.

Effect of interaction with heavy metal

Heavy metal ions were prepared in the laboratory by dissolving their salts in water to form aqueous solutions. The salts include; copper sulfate, mercury oxide, zinc oxide, ferrous chloride, lead oxide and silver nitrate. 5 ppm of each of the metal ions was separately added into the MSM containing glyphosate. The procedure was as described above.

Screening glyphosate as an electron donor source for molybdenum reduction

The low phosphate medium (LPM) was prepared by dissolving the following into one liter of distilled water; 3 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4.7H_2O$, 5 g of NaCl, 2.42 g of $Na_2MoO_42H_2O$, 0.71 g of Na_2HPO_4, 0.5 g of yeast extract and 1 g of glyphosate was used as the carbon source, with glucose as control. The pH of the medium was adjusted to 7.5 and then autoclaved at 121 ºC, 115 kPa for 15 min, glyphosate was autoclaved separately and afterwards added to the medium after cooling. 10 mL of the LPM was dispensed into the Vijou bottles and inoculated with the bacterial isolate. Absorbance at 865 nm was taken as 0 h, 24 h, 48 h, 54 h and 72 h for Mo-blue production.

Statistical analysis

All experiments were conducted in triplicate. Experiments errors were shown as bars of standard deviation. All data were statistically analyzed using GraphPad Instat. One-way ANOVA (95% confidence interval) was considered as statistical significance.

RESULTS AND DISCUSSION

Screening of the Isolates

Of the seven (7) molybdenum-reducing isolates screened for glyphosate degradation, isolate A (*Pseudomonas* sp.) grew faster and showed the highest colony when incubated for 72 h at 37 °C (**Table** 1).

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Table 1. Screening of glyphosate-degrading bacteria.

Isolates	Colony count (CFU/mL)
Isolate A	198 × 10^6
Isolate B	98 × 10^6
Isolate C	71 × 10^6
Isolate D	65 × 10^6
Isolate E	58 × 10^6
Isolate F	96 × 10^6
Isolate G	129 × 10^6

Characterization of glyphosate degradation

Effect of incubation time

From the result as presented in **Fig. 1**, it was shown that the bacteria grew best after 48 h of incubation, with a significant ($p<0.05$) decline in growth above this optimum condition.

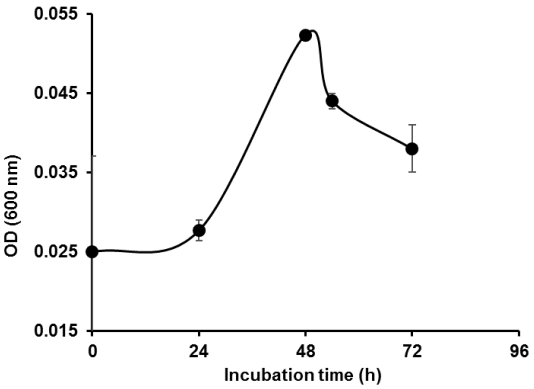

Fig 1. Effect of incubation time (h) on the growth of glyphosate-degrading *Pseudomonas* sp. Error bars represent mean ± standard deviation (n=3).

Effect of glyphosate concentration (carbon source) on glyphosate reduction

The effect of carbon source on glyphosate reduction in this bacterium was initially evaluated at different ranges, 0.5 – 10 g/L. The result shows that 1.0 g/L glyphosate (as carbon sole source) was the optimum for bacterial growth as shown in **Fig. 2a** below, and no statistical difference ($p>0.05$) exist between 1.0 g/L and 1.5 g/L. However, the effect of concentration over time revealed that 2 g/L was the optimum after 48 h of incubation, and a sharp decline in growth was observed at all concentrations above this incubation period (**Fig. 2b**).

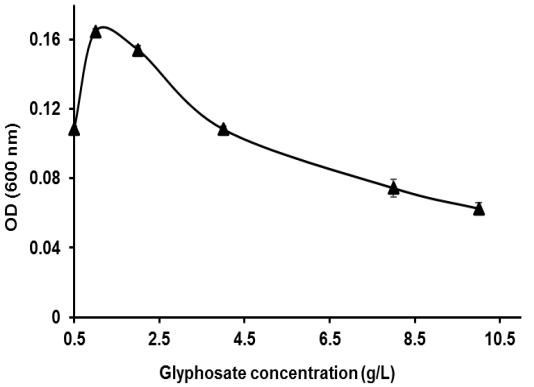

Fig 2a. Effect of glyphosate (carbon source) concentrations on the growth of glyphosate-degrading *Pseudomonas* sp. Error bars represent mean ± standard deviation (n=3).

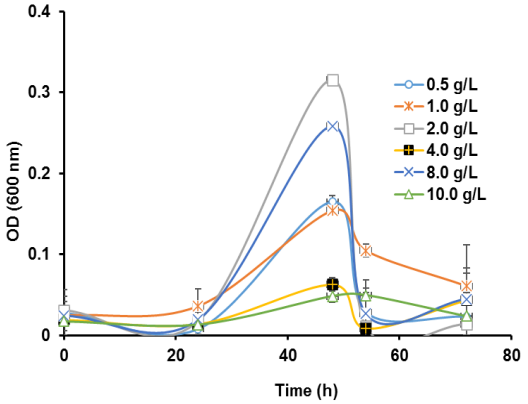

Fig 2b. Effect of various glyphosate concentrations per time of incubation on the growth of glyphosate-degrading *Pseudomonas* sp. Error bars represent mean ± standard deviation (n=3).

Effect of inoculum size on Glyphosate biodegradation

The effect of inoculum size on glyphosate reduction was evaluated at different inoculum sizes, ranging from 50 µL – 1000 µL. The result shows that the optimum inoculum was between 400 and 600 µL, with no significant difference ($p>0.05$) between these optimum ranges within 24 h of incubation (**Fig. 3a**). However, the effect of inoculum over the incubation period revealed that 400 µL inoculum was best after 48 h of incubation.

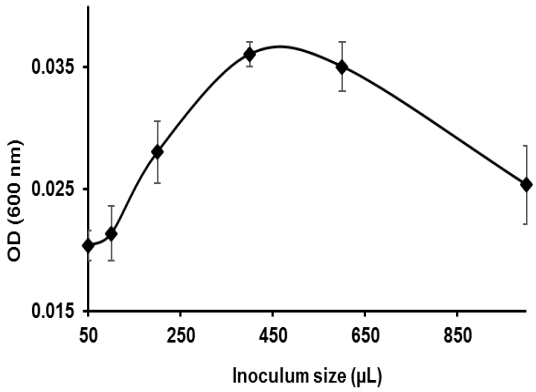

Fig 3a. Effect of inoculum sizes (µL) on the growth of glyphosate-degrading *Pseudomonas* sp. The error bar represents the mean ± standard deviation (n=3).

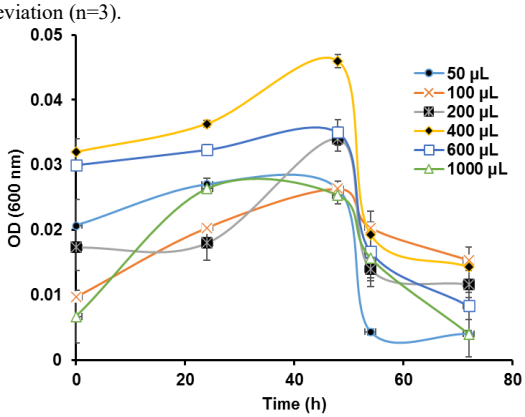

Fig 3b. Effect of various inoculum sizes (µL) per time of incubation on the growth of glyphosate-degrading *Pseudomonas* sp. Error bars represent mean ± standard deviation (n=3).

Effect of initial pH

Different pH ranges of 5.5 – 8.5 were used to evaluate its effect on glyphosate degradation. The result shows that after 24 h of incubation, the optimum bacterial growth was at pH 7.0, with a

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significant reduction ($p<0.05$) in growth at pH above the optimum value as seen in (**Fig. 4a)** below. The isolate showed better growth and degradation of glyphosate at a slightly acidic pH than alkaline (pH 7.5 – 8.5). Similarly, the effect of pH throughout incubation revealed that pH 7.0 was the best irrespective of the incubation period (**Fig. 4b**).

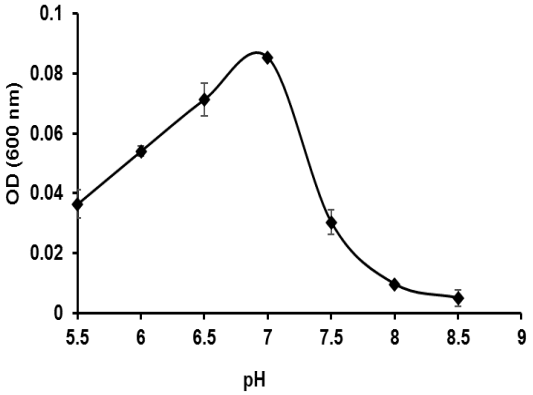

Fig 4a. Effect of initial pH on growth of glyphosate-degrading *Pseudomonas* sp. The error bar represents the mean ± standard deviation (n=3).

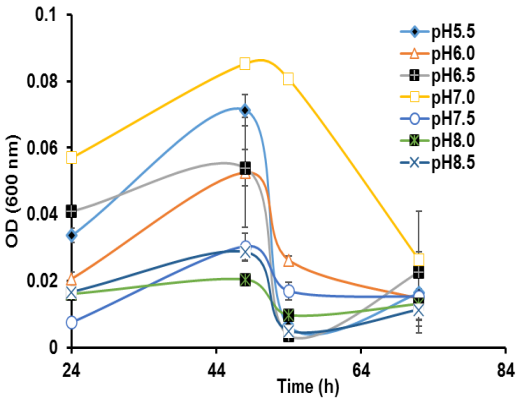

Fig 4b. Effect of various initial pH per time of incubation on the growth of glyphosate-degrading *Pseudomonas* sp. Error bars represent mean ± standard deviation (n=3).

Effect of temperature

The effect of temperature on glyphosate biodegradation was studied at temperatures 25, 30, 35, 40 and 45 ºC. The result shows that the optimum temperature was 40 ºC, and a significant ($p<0.05$) growth decline was noticed at a temperature beyond the optimum (**Fig. 5a**). Interestingly, this temperature was best even after the 48 h incubation period (**Fig. 5b**).

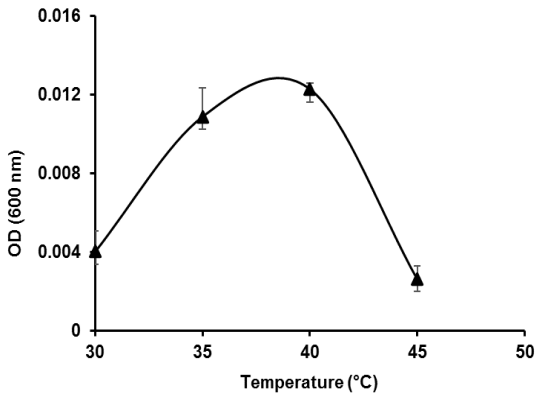

Fig 5a Effect of temperature on growth of glyphosate-degrading *Pseudomonas* sp. after 48h of incubation. The error bar represents the mean ± standard deviation (n=3).

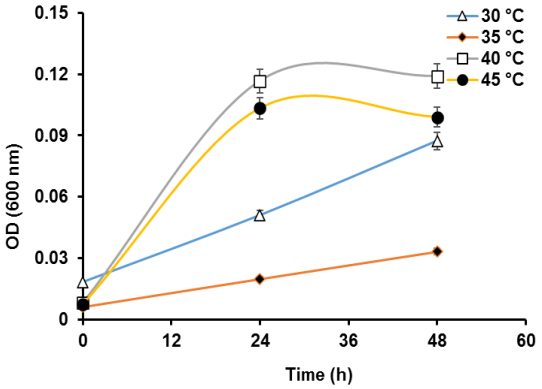

Fig 5b. Effect of various temperatures over time on growth of glyphosate-degrading *Pseudomonas* sp. Error bar represents mean ± standard deviation (n=3).

Effect of agitation

The effect of aeration on glyphosate degradation was evaluated by incubating the culture under shaking and static conditions. Growth of this isolate was found to be favored by shaking in all the incubation periods studied, with a significant difference ($p<0.05$) between 0 h < 24 h < 48 h (**Fig. 6**).

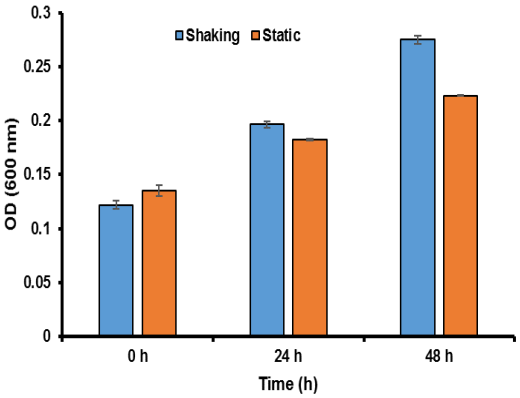

Fig 6. Effect of aeration on the growth of glyphosate-degrading *Pseudomonas* sp. after 48h of incubation. Error bars represent mean ± standard deviation (n=3).

The effect of heavy metals on the growth of glyphosate-degrading isolate

The effect of various interacting metals on glyphosate degradation was evaluated by dissolving their salts of mercury oxide, zinc oxide, copper sulfate, ferrous chloride, lead oxide and silver nitrate. The result shows that all the tested heavy metals significantly inhibited glyphosate degradation in the order of Cu>Zn>Pb>Hg>Ag>Fe compared to the control (**Fig. 8**).

Glyphosate as a carbon source for molybdenum bioreduction

As this isolate was earlier used for molybdenum reduction [10], the potential utilization of glyphosate as a source of carbon and electron donor to reduce hexavalent molybdenum to molybdenum blue (Mo-blue) was studied in low phosphomolybdate media (LPM), with glucose as control. Measuring the molybdenum blue (Mo-blue) produced at 865 nm, indicated glyphosate is a poor electron donor source to facilitate molybdenum reduction when compared to glucose (control)

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which shows approximately 3-fold higher ($p<0.05$) Mo-blue production than glyphosate (**Fig. 8**).

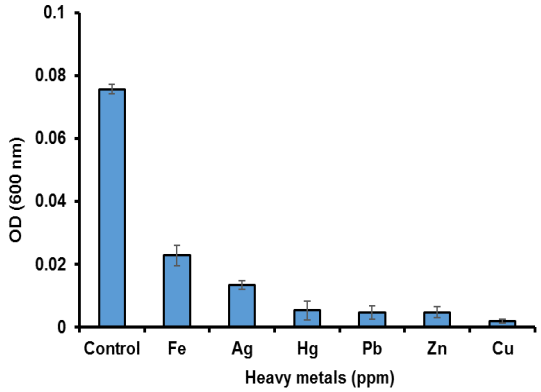

Fig 7. Effect of various heavy metals on the growth of glyphosate-degrading *Pseudomonas* sp. The error bar represents the mean ± standard deviation (n=3).

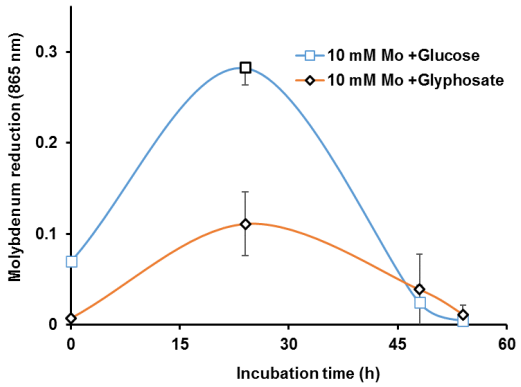

Fig 8. Glyphosate as carbon and electron donor source for molybdenum reduction in LPM over an incubation period of 48 h with glucose as control. Error bars represent mean ± standard deviation (n=3).

DISCUSSION

It was not until recently that researchers became interested in applying glyphosate-degrading bacteria for the bioremediation of polluted soils. The first attempt to apply the laboratory strain *Pseudomonas* sp. 4ASW, which is capable of cleaving glyphosate with the production of sarcosine, was unsuccessful because its C–P lyase was completely inactivated under field conditions [8]. An important advantage of the introduced strains was their ability to utilize glyphosate completely, making it possible to avoid an accumulation of toxic intermediates. The effect of incubation time is important as it is an act of maintaining controlled environmental conditions to favour the growth or development of microbes or to maintain optimal conditions for a biological reaction. The significant difference observed (**Fig. 1**) could be a result of the bacteria reaching its stationary phase.

Microorganisms like other organisms need nutrients to survive and be viable, and to carry out metabolic processes. Their ability to utilize a minimal amount of carbon source for their growth may vary and may depend on their ability to be able to utilize it. In this study, other carbon sources were not utilized to compare if glyphosate degradation would still take place in their presence of them. Glyphosate as a carbon source in the absence of any other carbon source showed an optimum degradation at 1.0 g/L. *Acetobacter* sp. and *P. fluorescens* were reported to have optimal growth at 7500 ppm with both strains able to tolerate up to 250,000 ppm of glyphosate [11].

Determining the effect of the inoculum size for optimum glyphosate reduction is important in the aspect that a high concentration of inoculum has been shown to inhibit the growth of the bacterium (**Fig. 3a**) thereby, inhibiting glyphosate degradation. The amount of inoculum used is important for the standard growth of bacterial isolate, a low inoculum might cause substrate depletion because the concentration of the nutrient is higher than the number of bacteria in the medium. While a high concentration may lead to death and growth inhibition because probably the concentration of nutrients in the medium is lower and insufficient feeding would lead to inhibition of growth [12].

pH maintenance is very crucial in bacterial medium as it affects the growth and proliferation of cells. Lowest growth was found at pH between 8.0 – 8.5, and the highest was found at pH 7.0 (**Fig. 4a**). Glyphosate degradation was not supported at pH below 5.5, which is probably due to inhibition of bacterial growth. Since pH is a measure of the degree of acidity, neutrality and alkalinity of a medium, bacteria like other microorganisms also prefer a fit and physiological pH to survive and carry out their metabolic processes [13]. The ability of these microbes to be viable in higher and even lower pH (i.e below 5.0) depends on their capability to maintain the difference in pH of their intracellular and extracellular environment [13]. *Bacillus cereus* CB4 [14] showed optimum glyphosate degradation at pH 6.0 – 7.0, while a more alkaline condition was preferred by *Pseudomonas putida*, with optimum growth at pH 9.0 [15]. However, inadequate literature is available on glyphosate-degrading bacteria favoring acidic conditions since most glyphosate degraders prefer neutral-alkaline pH for optimum degradation rate [6].

Several bacteria have been reported to degrade glyphosate at various temperatures, but the preferable temperature is 30 ºC and 35 °C. *Ochobactrum* sp. GDOS [16] and Pseudomonas putida [15] have the highest bacterial growth at 30 °C, suggesting extensive utilization of glyphosate by these bacteria. The reason for the high preferred temperature by *Pseudomonas* sp. in this study (**Fig. 4.5a**) could be a result of the high temporal region in which this bacterium was isolated.

The effect of shaking is a meticulous and important aspect of bacterial growth and degradation, it not only improves mass and oxygen transfer between the different phases but also maintains homogenous chemical and physical conditions in the medium by continuous mixing [17]. On the other side, agitation can cause sheer forces which influence microorganisms in several ways, such as changes in morphology, variation in growth and metabolite formation, aeration determines the oxygenation of the process, and also contributes to the mixing of the medium, especially where mechanical agitation speeds are low [17]. The high growth increment observed in (**Fig. 6**) is a result of the mechanical agitation rate and oxygenation process as described above.

Metal toxicity is of great environmental concern because of its bioaccumulation and non-biodegradability in nature [18]. Several inorganic metals like magnesium (Mg), nickel (Ni), chromium (Cr), copper (Cu), calcium (Ca), manganese (Mn), and sodium (Na) as well as zinc (Zn) are vital elements needed in small quantity for metabolic and redox functions. Heavy metals such as aluminium (Al), lead (Pb), cadmium (Cd), gold (Au), mercury (Hg), and silver (Ag) do not have any biological role and are toxic to living organisms. Checking the possible interference

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of these metals with glyphosate in the pollution site is of great importance. The ability of Pseudomonas sp. to tolerate and degrade glyphosate even in the presence of heavy metals is advantageous, though all the metals significantly inhibit the degradation. [9] were the first to report the use of molybdenum-reducing bacteria for glyphosate degradation, however, the utilization of glyphosate as an electron donor source for molybdate reduction was not reported in any organism, making this finding to be novel. The fact that glyphosate did not significantly support Mo-blue production in this bacterium when compared with glucose control is an indication that the bacterium prefers simple assimilable carbon sources as electron donors for the reduction of molybdenum to Mo-blue.

CONCLUSION

A bacterium previously isolated for molybdenum reduction has been characterized for glyphosate utilization as the sole source of carbon and possibly as an electron donor for molybdate reduction. The isolate degraded glyphosate optimally at pH 6.0, the temperature at 40 ºC, 1.0 g/L glyphosate concentration, and 100 µL inoculum when incubated for 48 h. This isolate was significantly affected by all the tested heavy metals. The bacterium prefers glucose as an electron donor for molybdenum reduction than glyphosate, even though its utilization is still novel. Work is still underway to further optimize the potential of this bacterium for future bioremediation.

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