BESSM, 2023, Vol 7, No 1, 38-43
https://doi.org/10.54987/bessm.v7i1.901

HIBISCUS PUBLISHER

BULLETIN OF ENVIRONMENTAL SCIENCE & SUSTAINABLE MANAGEMENT

Website: http://journal.hibiscuspublisher.com/index.php/BESSM/index

BESSM VOL 7 NO 1 2023
Azokeratin

Characterization of a Glyphosate-degrading Bacterium from a Paddy Field in Kepala Batas, Penang

Motharasan Manogaran[1], Hafeez Muhammad Yakasai[2] and Mohd Yunus Shukor[3,4]*

[1]Malaysia Genome and Vaccine Institute (MGVI) National Institute of Biotechnology Malaysia (NIBM) Jalan Bangi, 43000 Kajang, Selangor, Malaysia.
[2]Department of Biochemistry, Faculty of Basic Medical Sciences, College of Health Science, Bayero University Kano, P. M. B 3011, Kano State-Nigeria.
[3]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[4]Agribiotechnology Group, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia.

*Corresponding author:
Mohd Yunus Shukor,
Department of Biochemistry,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia,
43400 UPM Serdang,
Selangor,
Malaysia.
Email: mohdyunus@upm.edu.my

HISTORY

Received: 15th April 2023
Received in revised form: 25th June 2023
Accepted: 15th July 2023

KEYWORDS

Bioremediation
Glyphosate
Pollution
Pseudomonas sp.
Heavy metal

ABSTRACT

Bioremediation of contaminants, including glyphosate, a herbicide, is an economically viable and environmentally friendly technique. Glyphosate is the most utilized herbicides for weed management. Pollution from glyphosate is dangerous to wildlife and their habitats. Soil *Pseudomonas* sp. strain UMP-KB2 obtained from a paddy field was used as the only source of carbon and described for its capacity to degrade glyphosate. The growth of these bacteria was measured spectrophotometrically as A600 nm in response to changes in incubation time, g inoculum size, glyphosate concentration (carbon source), temperature and pH. The bacterium degrades glyphosate optimally at pH 7.0, glyphosate concentration of 0.5 g/L, temperatures of between 30 and 35 ºC, and inoculum size 1% (v/v). Growth at 0.5 g/L glyphosate shows a two-day lag period and nearly 90% degradation after six days of incubation. The isolation of a glyphosate-degrading bacterium that utilizes glyphosate as a carbon source will be very useful in mineralizing glyphosate in contaminated agriculture soil.

INTRODUCTION

Environmental contamination is reaching frightening levels worldwide. Energy use and garbage production have both increased as a result of urbanization, industrialization, and overall economic growth. Greenhouse gas emissions, acid deposition, water pollution, improper waste disposal, and other forms of environmental degradation are all recognized as threats to human health on a global scale and warrant further study from a variety of disciplinary and methodological perspectives, including those of public health, public policy, environmental engineering, and public health promotion and disease prevention [1–4]. Early in life, exposure to environmental pollutants can cause a wide range of negative health outcomes, including but not limited to: infant mortality, perinatal disorders, respiratory disorders, allergies, cancers, cardiovascular disease, elevated oxidative stress, endothelial dysfunction, psychological disorders, and many others. Exposure to environmental particulate matter has been associated in numerous studies to an increased risk of disease, organ disruption, cancer, and other chronic diseases, as well as an increased chance of mortality [5–10].

Glyphosate is a systemic herbicide that is non-selective. It is utilized to control most seasonal and perennial plants. There is growing public safety concern over the presence of glyphosate metabolites in these goods. However, there is scant data supporting the carcinogenicity of glyphosate human exposure, despite the fact that studies on animals indicated that high doses of the chemical caused harm to organs, reproduction, and the nervous system. Glyphosate has been found in water and other environmental samples mostly because it is widely used in farming. As more and more research shows that glyphosate is dangerous to living things, the question of how to stop it from building up in the first place (in soils and water supplies) and how to get rid of it once it does is gaining attention [11–18].

It is estimated that annually between 125,000 and 130,000 metric tons of herbicides are used around the world. Over 18 countries have outright banned glyphosate from agricultural use. Thousands of farmers in Malaysia continue to use this chemical without proper safety equipment. Due to the concern about its toxicity, glyphosate bioremediation has been recommended as an alternative to the physical and chemical methods of glyphosate degradation [19–22]. In archaea, bacteria, Apicomplexa, algae, fungus, and plants, glyphosate suppresses the 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase that is involved in the shikimate pathway. EPSP is an essential precursor for the de novo synthesis of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan as well as the vitamins folic acid and menaquinone. EPSP is produced by the EPSP synthase, which transform shikimic acid-3-phosphate (S3P) and the glycolytic intermediate phosphoenolpyruvate (PEP) into EPSP [23–26]. As bacteria that can utilize glyphosate as a carbon source is rare, the aim of this study is to screen for such bacteria from a paddy field that has a history on glyphosate applications for decades.

MATERIALS AND METHODS

Chemicals

All chemicals used in this work were of analytical grade. Glyphosate (N-(phosphonomethyl)glycine) was purchased as a technical grade chemical (95%, Zhengzhou Delong Chemical Co., Ltd . 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.

Soil sampling

Soil was sampled from a paddy field in Kepala Batas, Seberang Perai, Pulau Pinang, Malaysia in October 2022. Soil was sampled 5 cm from the topsoil using a sterile spatula and placed in sterile polycarbonate container.

Screening of glyphosate-degrading isolate

Soil sample (1 g) was added to 9 mL of sterile tap water and mixed. Then, 0.2 mL aliquot was transferred and streaked on a mineral salt agar medium (pH 7.5) 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). A stock solution of glyphosate (10 g/L) was prepared in deionized water, and the final concentration of glyphosate used as the only carbon source was 1 g/L. Glyphosate maximum solubility in deionized water was about 12 g/L. The medium was then autoclaved at 121 ºC, 115 kPa for 15 min, and glyphosate was added to the medium through filter sterilization (0.2 micron filter) at 60 °C. Two distinct colonies formed indicating the presence of glyphosate-degrading microorganisms. These colonies were restreaked on fresh agar medium. A single colony was transferred into 10 mL of glyphosate MS medium in 28 mL-universal bottles and incubated at room temperature for 3 d on an orbital shaker at 150 rpm. The best glyphosate-degrading bacterium based on A600 nm measurement was utilized for further optimization.

Characterization of glyphosate degradation

Experiments were carried out using a microplate titer approach. The glyphosate-MSM medium above was pipetted into the microplate (200 µL) and 20 µL bacteria inoculum was mixed into the wells of the microplate.

The microplate was then covered and incubated statically for 5 days at room temperature. 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). The characterization of the best isolate include studies on the effects of incubation time, glyphosate concentration, inoculum size, pH and temperature.

Determination of glyphosate using HPLC

The method of [27] was utilized with slight modification using an isocratic gradient eleution. An Agilent 1200 series equipped with an autosampler, and a UV detector was utilized in this study. The column was a Zorbax Agilent SAX, 4.6 mm ID x 250 mm (5 µm) column. The mobile phase consisted of 6.2 mM KH_2PO_4 in 4% (v/v MeOH, with the pH adjusted to 2.1 with 85% phosphoric acid. The flow rate was 1 mL/min and the detector was set at 195 nm.

Morphological, physiological and biochemical characterization

The bacterium was biochemically and phenotypically characterized using standard morphological and biochemical methods according to the Bergey's Manual of Determinative Bacteriology [28]. Interpretation of the results was carried out via the ABIS online system [29].

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

The screening yielded two distinct colonies of bacterium able to utilize glyphosate as a carbon source. The best isolate based on a higher absorbance value at A600 nm was chosen for partial identification.

Partial identification of the bacterium

The bacteria was a Gram-negative, rod-shaped, motile microorganism. Culture, morphology, and a battery of biochemical analyses all pointed to the same bacterium, which allowed for its positive identification (**Table 1**) to the Bergey's Manual of Determinative Bacteriology [28] and using the ABIS online software [29]. The software gave two suggestions for the bacterial identity with similar homology (80%) and accuracy (84%). They were *Pseudomonas putida* or *P. plecoglossicida*. This bacterium was tentatively identified as *Pseudomonas* sp. strain UMP-KB2. Numerous bacteria from this genus is known for their ability to degrade pesticides including glyphosate [26,30–37]. Hence, at this juncture, the assignment to the species level cannot be done. More works in the future especially molecular identification technique through comparison of the 16srRNA gene are needed to identify this species further. Other glyphosate-degrading bacterium includes *Alcaligenes* sp. [38], *Flavobacterium* sp. [39], *Bacillus megaterium* [36], *Geobacillus caldoxylosilyticus* [40], Enterobacter cloacae [41], *Rhizobium* sp. and *Agrobacterium* sp. [42] and *R. aquatilis* [43].

Table 1. Biochemical tests.

Motility	+	Utilization of:	
Hemolysis	+	L-Arabinose	+
Growth at 4 ºC	–	Citrate	+
Growth at 41 ºC	+	Fructose	+
Growth on MacConkey agar	–	Glucose	+
Arginine dihydrolase (ADH)	+	meso-Inositol	d
Alkaline phosphatase (PAL)	+	2-Ketogluconate	+
Indole production	d	Mannose	+
Nitrates reduction	–	Mannitol	–
Lecithinase	–	Sorbitol	–
Lysine decarboxylase (LDC)	–	Sucrose	+
Ornithine decarboxylase (ODC)	–	Trehalose	–
ONPG (beta-galactosidase)	–	Xylose	–
Esculin hydrolysis	–		
Gelatin hydrolysis	d		
Starch hydrolysis	–		
Urea hydrolysis	+		
Oxidase reaction	+		

Note: + positive result, − negative result, d indeterminate result

Characterization of glyphosate degradation

Effect of pH

The bacterium thrived effectively at pH 7.0. It seems that growth was severely stunted at pH levels above 9 and below 5. This range, especially around neutral, is ideal for most glyphosate-degrading bacteria (**Fig. 1**).

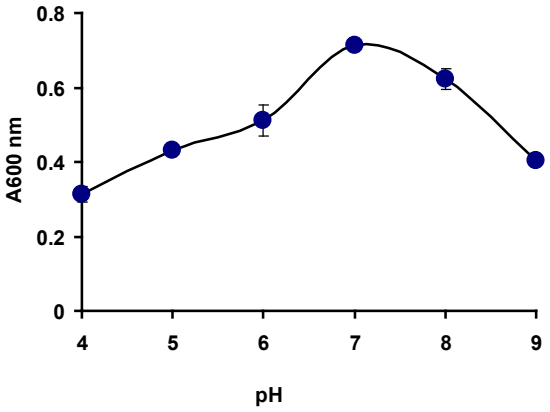

Fig 1. Effect of pH on the growth of glyphosate-degrading *Pseudomonas* sp. strain UMP-KB2. Error bars represent mean ± standard deviation (n=3).

Effect of glyphosate concentration on glyphosate reduction

From concentrations of 0.1 to 1 g/L, the impact of carbon source on this bacteria's ability to degrade glyphosate was measured. Results reveal that at values above 0.5 g/L, growth was substantially reduced when glyphosate was the only source of carbon (**Fig. 2**).

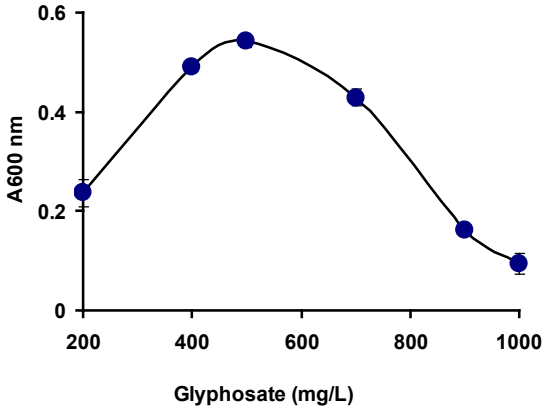

Fig 2. Effect of glyphosate concentration on glyphosate degradation by *Pseudomonas* sp. strain UMP-KB2. Error bars represent mean ± standard deviation (n=3).

Effect of inoculum size on Glyphosate biodegradation

Different inoculum sizes, from 0.1 to 1 percent (v/v) from an initial stock of A600 nm of 1.0, were tested to determine their impact on glyphosate reduction. The data reveal that as the size of the inoculum was raised, the optimal inoculum also grew (**Fig. 3**).

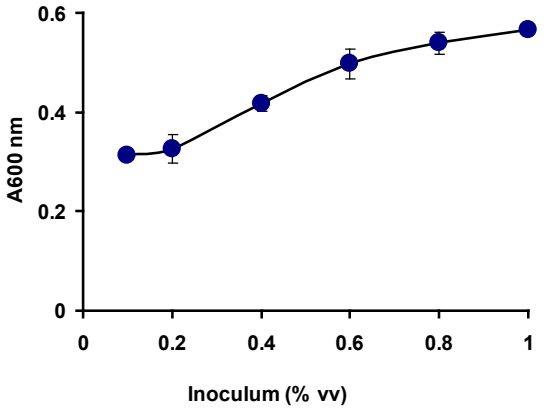

Fig 3. Effect of inoculum sizes (% v/v) on the growth of glyphosate-degrading *Pseudomonas* sp. strain UMP-KB2. The error bar represents the mean ± standard deviation (n=3).

Effect of initial pH

The impact on glyphosate breakdown in the pH range of 5.5–8.5 was measured. After 4 days of incubation, the results demonstrate that pH 7.0 is optimal for bacterial growth, with growth significantly reduced ($p<0.05$) at values above the optimal value (**Fig. 4).**

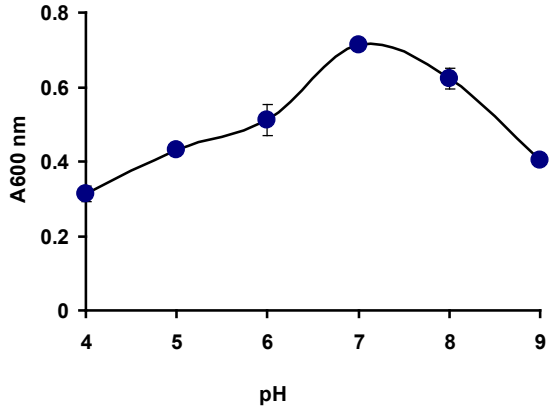

Fig 4. Effect of initial pH on glyphosate degradation by *Pseudomonas* sp. strain UMP-KB2. The error bar represents the mean ± standard deviation (n=3).

Effect of temperature

Biodegradation of glyphosate was investigated at temperatures between 25 and 50 °C. The results suggest that the optimal temperature ranged from 30 to 35 °C, with a statistically significant ($p<0.05$) decrease in growth observed at higher temperatures (**Fig. 5**).

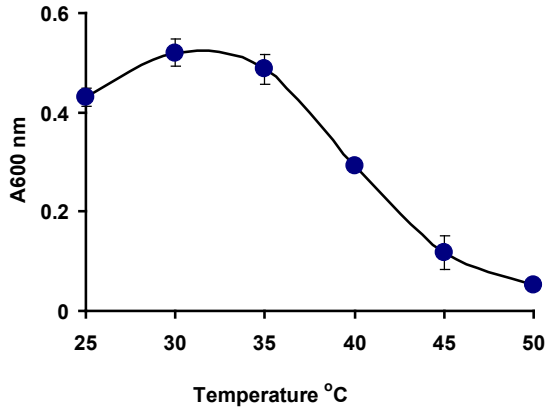

Fig 5. Effect of temperature on glyphosate degradation by *Pseudomonas* sp. strain UMP-KB2. The error bar represents the mean ± standard deviation (n=3).

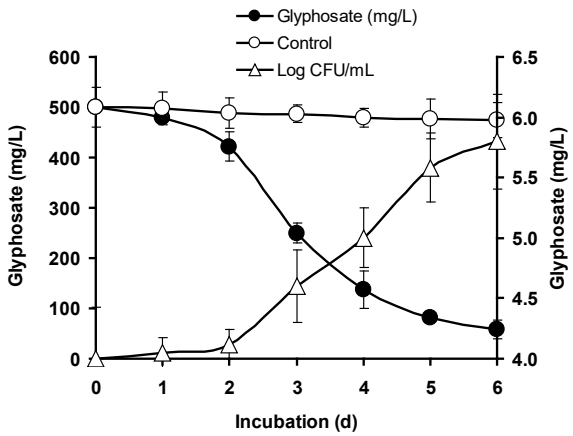

Fig. 6. Growth profile of *Pseudomonas* sp. strain UMP-KB2.on 500 mg/L glyphosate. Each data point represents the mean ± standard deviation of three replicates.

Growth of bacterium and degradation of glyphosate

The growth of this bacterium at 500 mg/L glyphosate shows a lag period that ranges from 4 to 8 hr (**Fig. 6**). Glyphosate concentration was decreases concomitant to cellular growth. Abiotic degradation of glyphosate was minimal as judged by the control.

DISCUSSION

The use of glyphosate-degrading bacteria in the bioremediation of contaminated soils has only lately piqued the interest of scientists. Because its C-P lyase was entirely inactivated under field conditions, the initial attempt to utilize the laboratory strain *Pseudomonas* sp. 4ASW, which can cleave glyphosate with the synthesis of sarcosine, was unsuccessful [44]. The capacity of the added strains to completely metabolize glyphosate meant that harmful intermediates might be avoided. Incubation is the practice of keeping a specific set of environmental parameters constant in order to promote the growth or development of microorganisms or to keep the conditions appropriate for a biological reaction.

Like all living things, microorganisms require nutrients to maintain their health and viability and to fuel their metabolic functions. Their growth potential, as well as their capacity to use a low-carbon source, may be inversely proportional. This study did not test whether or not glyphosate degradation would proceed in the presence of other carbon sources. In the absence of any other carbon source, optimal breakdown of glyphosate (a carbon source) occurred at a concentration of 1.0 g/L. Both *Acetobacter sp.* and *P. fluorescens* were shown to grow best at 7500 ppm (7.5 g/L), despite being able to survive concentrations of glyphosate as high as 250,000 ppm (250 g/L) [45].

Because it has been shown that a high concentration of inoculum can hinder the growth of the bacterium and, as a result, glyphosate breakdown, it is essential to know the effect that the size of the inoculum has on the level of glyphosate reduction that can be achieved. You have to use the appropriate quantity of bacterial inoculum if you want your bacterial isolate to grow in a typical manner. If you don't use enough, the nutrients in the medium will be depleted before the bacteria have a chance to consume them, and the experiment will fail. On the other hand, a high concentration may cause death and growth inhibition due to the anticipated low concentration of nutrients in the medium, which would lead to inadequate nutrition and, as a result, stunted development. This would be the case if the medium had a high concentration [46].

The maintenance of a medium's pH is crucial because it affects cell growth and proliferation in bacterial settings. At pH values below 5, glyphosate degradation was not maintained, probably because acidic conditions are not conducive to bacterial development. Bacteria, like other microbes, demand a proper and physiological pH in order to survive and carry out their metabolic operations, and pH is a measure of the degree to which a medium is acidic, neutral, or alkaline. Because the pH scale quantifies how acidic, neutral, or alkaline a given medium is [47]. Their ability to regulate the pH gradient between their intracellular and external environments is crucial to their survival at pH values above and below 5.0 [48]. *Bacillus cereus* CB4 [49] demonstrated optimal glyphosate breakdown at pH 6.0 – 7.0, whereas *Pseudomonas putida* favored an environment with a higher alkaline concentration., with optimum growth at pH 9.0 [35].

However, there is a lack of published research on glyphosate-degrading bacteria that thrive in acidic environments. This is because the majority of glyphosate degraders require a pH range of neutral to alkaline for the best breakdown rate [39,42,50–57] including several glyphosate-degrading *Pseudomonas* spp. [26,30–37]

There have been reports of many bacteria that can breakdown glyphosate at a variety of temperatures; nonetheless, the optimal temperature range is between 30 ºC and 35 °C. *Ochrobactrum* sp. GDOS [52] and *Pseudomonas putida* [35] have the highest bacterial growth at 30 °C, pointing to a significant consumption of glyphosate on the part of these microorganisms. It is possible that the high temperature area in which this bacterium was isolated is related to the reason why it has such a high preferred temperature in our investigation. Most of the glyphosate degraders are mesophilic microorganisms and the temperature range for optimal growth varies between 25 to 35 °C [39,42,50–57] and also including several glyphosate-degrading *Pseudomonas* spp. [26,30–37]. Geobacillus caldoxylosilyticus [40] is thermophilic glyphosate-degrading bacterium which as an optimum temperature at 60 °C.

The isolation of a *Pseudomonas*-degrading glyphosate is not unique to this study as many bacteria of this genus have been known to degrade glyphosate. that the bacteria belonging to the genus *Pseudomonas* are the ones that most frequently breakdown glyphosate in the laboratory including several glyphosate-degrading *Pseudomonas* spp. [26,30–37]. Growth of these bacteria on glyphosate is because of their resistant towards the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (EPSPS) through gene mutation and duplication [25].

The small number of strains that were isolated from the medium that included glyphosate as the only source of carbon or phosphorus is in keeping with several publications that demonstrated a considerable drop in the population of microorganisms when glyphosate was introduced to the medium culture. This conclusion can be understood by the toxicity of artificial medium owing to the mode of action of glyphosate, which is to impede the shikimic acid pathway in order to achieve its desired effect. Glyphosate renders an organism unable to produce necessary aromatic amino acids, which ultimately results in cell death. This is due to the fact that the pathway for shikimic acid is present in virtually all microorganisms.

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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