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Identification of Promising Glyphosate-Degrading Bacteria Isolated from the Rhizosphere of Local Chili Pepper Plantation

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Abstract

The glyphosate's continuous application in agricultural fields has caused adverse environmental effects. Utilizing indigenous microorganisms as glyphosate-degrading agents can be an effective and eco-friendly solution. Isolate Cf2, obtained from a local chili pepper plantation in Indonesia, grew as a co-dominant isolate in media with 50 ppm glyphosate, indicating its potential as glyphosate-degrading bacteria. However, this isolate cannot be applied yet because it has not been identified. Identification can provide initial insights into the safety of indigenous isolates for ecological application. Thus, this study aims to identify isolate Cf2 by utilizing the 16s rRNA gene sequence as the genetic marker. The data was compared to the database using the web-based BLAST2 program (version 2.13.0). Then, the evolutionary relationship of this isolate with its closest relatives was assessed through phylogenetic tree reconstruction. The results demonstrate that the isolate Cf2 has a sequence similarity of 98.88% with Bacillus subtilis. The phylogenetic tree reconstruction further indicates that isolate Cf2 forms a monophyletic clade with this species. Hence, it can be concluded that isolate Cf2 is indeed B. subtilis. This study is the first report of glyphosate-degrading B. subtilis isolated from chili pepper plantations in Indonesia, offering a new insight into bioremediation strategies.

Keywords: B. subtilis, glyphosate, biodegradation, species, 16s rRNA gene

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INTRODUCTION

Glyphosate, also known as isopropylamine salt of N-phosphonomethyl-glycine, is the active ingredient in a non-specific organophosphate herbicide manufactured by Monsanto (Badani et al., 2023; Zhan et al., 2018). Glyphosate can be used in forestry and agriculture to control grasses and weeds by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is involved in the production of aromatic amino acids in plants and microorganisms, leads to their death (Feng et al., 2020; Zhan et al., 2018). It is widely utilized in almost all plant cultivation, including plantations, food crops, and horticulture (Nguyen et al., 2022). Glyphosate has an annual usage of about 600–750 thousand tonnes, which is expected to rise to 740–920 thousand tonnes by 2025 (Maggi et al., 2020).This widespread use underscores the urgent need to address concerns about its toxicity and potential health effects.

Over the past few decades, the extensive use of glyphosate has led to its widespread presence in the environment, posing a threat to humans and ecosystems. Despite over 20 nations having banned its agricultural use due to these concerns (Rusnam et al., 2023), many farmers, particularly in developing countries like Indonesia, continue to use it without adequate

protection, thus risking their health. Improper use of glyphosate can lead to health issues for users, including eye irritation, blurred vision, skin irritation, nausea, sore throat, difficulty breathing, headaches, nosebleeds, and possibly acute cancer (Feng et al., 2020; Firdous et al., 2020; Sun et al., 2019; Zhang et al., 2019). Glyphosate has also been found to be toxic to aquatic life (Zhang et al., 2024). Furthermore, several studies have shown that the biological activity of soils decreases for a considerable period after herbicidal treatment (Kulikova et al., 2020).

Glyphosate persists between 55 days to 3 years in the environment and adversely affects mammals, humans, and soil microbial ecosystems (Andriani et al., 2017; Badani et al., 2023; Maggi et al., 2020; Pileggi et al., 2020; Zhang et al., 2024). Due to its high solubility in water and ability to move easily, glyphosate can quickly seep into the soil, contaminating groundwater and accumulating in plant tissues (Zhan et al., 2018). Although glyphosate is rapidly absorbed in the soil through a ligand exchange mechanism, where it binds to soil oxides and clay minerals, recent studies have indicated a risk of soil phytotoxicity (Kulikova et al., 2020). Therefore, developing methods for enhancing glyphosate degradation in soil is crucial.

Glyphosate can either be degraded by biotic or abiotic means. There are several methods for degrading glyphosate, including adsorption, photocatalytic degradation, oxidation (using chlorine, permanganate, air, or ozone), filtration and flocculation, microbial degradation, and thermolysis (Nguyen et al., 2022; Zhan et al., 2018). Among these methods, microbial degradation is considered as the most effective and eco-friendly (Feng et al., 2020; Ibrahim et al., 2023; Kryuchkova et al., 2014). Various microorganisms can use glyphosate as a sole source of phosphorus, carbon, and nitrogen during its degradation.

Microorganisms primarily degrade glyphosate through enzymatic pathways. Bacteria belonging to the genera Escherichia, Pseudomonas, Agrobacterium, Klebsiella, Arthrobacter, Bacillus, and Rhizobium, as well as basidiomycete and ascomycete fungi, possess this capability (Badani et al., 2023; Ermakova et al., 2008; Zhan et al., 2018). In addition, indigenous bacteria isolated from areas contaminated with organophosphates have been found to be more effective in the degradation process (Ibrahim et al., 2023; Manogaran, Shukor, et al., 2017).

Nikmah & Lisdiana (2024) also isolated glyphosate-degrading bacterial candidates from a local chili pepper farming area in Blitar, Indonesia, that had been using synthetic herbicides with a glyphosate content of 360 g/L for weed control over the past 15 years. Multiple isolates were obtained through the screening procedure, including the co-dominant Cf6 and Cf2. Isolate Cf6 and Cf2 demonstrated survival after exposure to glyphosate at concentrations up to 50 ppm during the screening process, indicating their promising potential as future indigenous glyphosate-degrading agents. However, the implementation of unidentified indigenous bacteria is highly discouraged. While isolate Cf6 has been identified as Klebsiella quasipneumonia (Maulida & Lisdiana, 2024), isolate Cf2, which is predicted to have similar potential, has not yet been identified. Despite that, K. quasipneumonia is known as a human pathogen, raising concerns about the further application of isolate Cf6. The identification results of isolate Cf6, which indicates the possibility of the isolate being unsafe for ecological applications, led the urges to identify another potential isolate, isolate Cf2, which also demonstrated survival and became one of the dominant isolates in media containing 50 ppm glyphosate. Based on Nikmah & Lisdiana (2024), isolate Cf2 has colony morphological characteristics of punctiform form, raised elevation, entire margin, white pigmentation, opaque optic, and smooth surfaces. Isolate Cf2 also has a bacillus cell shape with a diplobacil arrangement, measuring 2.5 microns, and is gram-positive, motile, and catalase-positive (Nikmah & Lisdiana, 2024).

Identification of potential indigenous isolates, such as isolate Cf2, is important to ensure their safety in field applications. Conducting identification before further analysis is also considered more efficient and cost-effective. It allows for the early detection of potential risk and offers insights into its possible degradation pathways. Prompt identification can be

performed through molecular techniques that utilize genetic markers, such as 16S rRNA gene sequences. This method is a reliable standard for prokaryote taxonomy, particularly bacteria. Even though, in certain cases, relying solely on 16s rRNA sequences may not be sufficient to distinguish certain species within a genus and need complementary methods to validate the results. Hence, this study focuses on identifying isolate Cf2 based on its 16s rRNA gene sequence similarity with the database and predicting its degradation pathways based on the species identified through this study before conducting further analyses, such as examining the degradation rate.

METHOD

Preparation of Isolate Cf2 cells

Isolate Cf2 was routinely grown on slant nutrient agar media (Merck) containing 10 ppm glyphosate herbicide and stored at 4 °C. The addition of 10 ppm glyphosate in stock culture media was intended to maintain their ability to degrade glyphosate.

To prepare the cells for DNA extraction, a loop of culture was taken from the stock culture of isolate Cf2 and inoculated into 3 mL of Luria Bertani media (Merck). Then, the cultures were incubated aerobically on a shaker at 200 rpm overnight at room temperature.

Total DNA extraction of Isolate Cf2

The total DNA of isolate Cf2 was extracted using a Bacterial DNA Preparation Kit (Jena Biosciences) from a previously prepared culture. The extraction was performed according to the manufacturer's manual. A total of 1000 µl of the bacterial culture was harvested and centrifuged at 10,000 *xg* for 1 minute, after which the supernatant was discarded. The pellet proceeded to the DNA isolation procedure, mainly consisting of cell lysis, column loading, washing, and elution.

Prior to the cell lysis procedure, the cell pellet was resuspended in 300 µl resuspension buffer along with 2 µl of lysozyme and incubated at 37 °C for one hour. This step aimed to optimize the lysis of Gram-positive bacteria. After incubation, the cell pellet was recollected through centrifugation at 10,000 *xg* for 1 minute. Next, 300 µl lysis buffer and 2 µl RNase were added to the cell pellet and homogenized for 30 seconds. Following this, 8 µl Proteinase K was added and homogenized before being incubated at 60 °C for 10 minutes. The suspension was then cooled for 5 minutes before 300 µl binding buffer was added and vortexed briefly. The tube was then placed on ice for 5 minutes and centrifuged at 10,000 *xg* for 5 minutes.

A spin column was placed into a 2 ml collection tube. Then, the supernatant, which separated from the lysate, was loaded into the spin column, centrifuged at 10,000 *xg* for 1 minute, and discarded the flow-through. Next, 500 µl of washing buffer was added to the spin column, centrifuged at 10,000 *xg* for 30 seconds, and then the flow-through was discarded. This washing step was done twice. After discarding the flow-through, additional centrifugation was done at 10,000 *xg* for 1 minute to remove any residual washing buffer. Subsequently, the 2 ml collection tube and the flow-through washing buffer residue were discarded, and the spin column was placed in a sterile 1.5 ml tube. A total of 40-50 µl of elution buffer was added to the spin column and incubated for 1 minute at room temperature. Subsequently, the sample was centrifuged at 10,000 *xg* for 2 minutes. The obtained total DNA was checked quantitively and qualitatively before being stored at -20 °C before the amplification procedure.

Amplification of 16s rRNA gene

The 16s rRNA gene of isolate Cf2 was amplified using the polymerase chain reaction (PCR) method. The PCR process consisted of 30 cycles, including 30 seconds of denaturation at 96°C, 30 seconds of annealing at 55°C, and 1.5 minutes of extension at 72°C. The procedure started with an initial pre-denaturation phase at 96°C for 2 minutes and concluded with a final extension phase at 72°C for 4 minutes. The total volume of the PCR reaction mixture was 25 µl, which comprised 1 µl of template DNA, 12.5 µl of 2x PCR master mix, 0.5 µl of 10 µM

universal primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.5 µl of 10 µM universal primer 1492R (5'-GTTTACCTTGTTACGACTT-3'), and 10.5 µl of nuclease-free water. To estimate the size of the amplified DNA, the amplicon was analyzed on a 2% agarose gel containing 0.5 mg/ml ethidium bromide, alongside a 1 kb DNA marker. The qualified amplicon was then sent to 1st Base Malaysia for sequencing analysis.

16s rRNA sequencing analysis

Single-pass DNA sequencing was performed using the Sanger DNA sequencing method, involving two reactions with primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), followed by capillary electrophoresis.

Consensus sequence

The contig process involved analyzing the forward and reverse sequence data using BioEdit software. A pairwise alignment of the forward and reverse sequences was performed before generating a consensus sequence, which was then saved in FASTA format.

Data analysis

The 16S rRNA sequence data from the isolate Cf2 was analyzed for similarity with the database (https://www.ncbi.nlm.nih.gov/) using the web-based BLAST2 program (version 2.13.0). A phylogenetic tree was then reconstructed using MEGA 11, employing the Neighbor-Joining method and bootstrap analysis with 1000 replicates. The Maximum Composite Likelihood genetic distance was used for this reconstruction (Hall, 2013). The phylogenetic analysis aims to illustrate the relationships among the test isolates and other species, highlighting their level of kinship from closest to most distant.

RESULTS AND DISCUSSION

The increased concerns regarding the long-term application of glyphosate in agricultural fields are caused by its persistent nature, which makes it challenging to degrade quickly. The slow degradation process of glyphosate may cause accumulation of this substance in the soil, resulting in adverse environmental impacts. Thus, the search for new biodegradation agents from contaminated locations is being conducted intensively. Several prospective bioremediation agents for glyphosate have been identified and studied. These include *Chryseobacterium* sp. Y16C, *Proteus mirabilis*, *Bacillus tropicus*, *Bacillus subtilis*, *Bacillus cereus*, *Enterobacter cloacae*, *Agrobacterium tumefaciens*, *Ochrobactrum pituitosum*, *Rhodococcus soli*, *Pseudomonas* spp., *Burkholderia vietnamiensis*, and *Burkholderia* sp. All of these species have been isolated, identified, and tested for their ability to degrade glyphosate (Andriani et al., 2017; Fan et al., 2012; Ibrahim et al., 2023; Kryuchkova et al., 2014; Manogaran, et al., 2017; Nguyen et al., 2022; Rossi et al., 2021; Rusnam et al., 2023; Yu et al., 2015; Zhang et al., 2022; Zhao et al., 2015).

Isolate Cf2, isolated from a local chili pepper farm in Blitar, Indonesia, has demonstrated the ability to degrade glyphosate. This capability was confirmed by its growth on minimum media containing 50 ppm glyphosate as the sole carbon source (Nikmah & Lisdiana, 2024). Identifying potential isolates before application in the fields is essential to ensure their safety to the environment and other living organisms. Molecular identification using 16s rRNA gene sequence as the genetic marker in this study revealed that isolate Cf2 is closely related to *Bacillus subtillis* and *Bacillus stercoris*, with a sequence similarity of 98.88% (Table 1). According to Yarza et al. (2014), isolates with a similarity percentage exceeding 98.7% can be classified within the same species. However, the results indicated that isolate Cf2 shares this similarity with two different species.

Bacillus is a diverse genus of bacteria which grow aerobically and form dormant endospores. Although *Bacillus* species were among the first bacteria ever characterized, their relationships with each other remain unclear and led to reclassification (Maughan & Van der Auwera, 2011) as in *B. stercoris* and *B. subtilis*.

Table 1. Closest relatives of isolate Cf2 based on its 16s rRNA gene sequence similarity

Isolate Code	Gram type	Closest strains	Sequence similarity*	Accession no. of the closest strains
Cf2	Positive	*Bacillus stercoris* strain D7XPN1	98.88% (1455)	NR_181952.1
		Bacillus subtilis strain DSM 10	98.88% (1517)	NR_027552.1
		Bacillus subtilis strain NRRL B-4219	98.88% (1168)	NR_116183.1
		Bacillus subtilis strain JCM 1465	98.88% (1472)	NR_113265.1
		Bacillus subtilis strain NBRC 13719	98.88% (1475)	NR_112629.1
		Bacillus subtilis strain BCRC 10255	98.88% (1468)	NR_116017.1

B. stercoris strain D7XPN1 is a bacterium isolated from a commercial food-waste degrading bioreactor that exhibits optimal growth at 45 °C (with a range of 24–50 °C) and pH of 7 (with a range of 5–9). This bacterium can thrive in environments with up to 7% NaCl. It is a facultative anaerobe by fermentation and forms white irregular colonies 1–2 mm in diameter when grown on dTSA. The cells are straight rods 4–5 µm long by 1 µm wide and stain Gram-positive (Adelskov & Patel, 2016). *B. stercoris* was initially classified as one of the subspecies of the *B. subtilis* known as *Bacillus subtilis* subs. *stercoris*. However, this subspecies was promoted to a new species based on comparative genomics, which was carried out to identify unique genes within each subspecies. These genes contribute to distinguishing phenotypes, such as producing different bioactive secondary metabolites. Notably, *B. subtilis* subs. *stercoris* conserves the genes required to produce fengycin and an unknown PKS/NRPS cluster, which are absent in *B. subtilis* subsp. *subtilis*. This distinctiveness prompted the reclassification of the subspecies into a new species (Dunlap et al., 2020).

This study employed the 16s rRNA gene sequence as a genetic marker, which has become a standard for prokaryotic taxonomy. The 16s rRNA gene comprises approximately 1,600 base pairs, divided into conserved regions and nine hypervariable regions (V1-V9). The conservative regions help determine the higher-ranking taxa, while the rapidly evolving regions help identify specific genera or species (Bukin et al., 2019). However, previous studies indicated that this genetic marker failed to distinguish *B. stercoris* from *B. subtilis* (Dunlap et al., 2020). Hence, the results obtained in this study, which showed the same similarity level of the test isolates with two species, *B. subtilis* and *B. stercoris*, were reasonable and consistent with the previous studies.

Based on the data in Table 1, isolate Cf2 showed a high similarity to six different species or strains. Therefore, phylogenetic analysis was conducted to reveal the evolutionary relationship of isolate Cf2 with its closest species. The phylogenetic tree illustrates the lines of evolutionary descent among various species, tracing back to a common ancestor. In other words, it visually represents the relationships between different organisms or species regarding their evolutionary timeline (Abaza, 2020; Hug et al., 2016).

In this study, phylogenetic analysis conducted using the Neighbor-Joining (NJ) algorithm and the Maximum Composite Likelihood genetic distance indicated that isolate Cf2 was evolutionarily closer to *B. subtilis* strain NBRC 13719 (accession # NR112629.1). This result was depicted in the visualization of the phylogenetic tree (Figure 1), which shows that isolate Cf2 forms a monophyletic clade with *B. subtilis* strain NBRC 13719, which is a collection of National Institute of Technology and Evaluation (NITE) Biological Research Center, Japan. *B. stercoris*, a close relative of isolate Cf2 based on BLAST analysis with the exact sequence

similarity percentage with *B. subtilis* (Table 1), interestingly forms a non-monophyletic clade with the target isolate. These findings suggest that isolate CF2 and *B. subtilis* share a more recent common ancestor compared to *B. stercoris*, which indicates a closer relationship between these two than isolate Cf2 to the latter species. Hence, based on the results of the BLAST analysis and the construction of the phylogenetic tree, it can be deduced that isolate Cf2 is *Bacillus subtilis*.

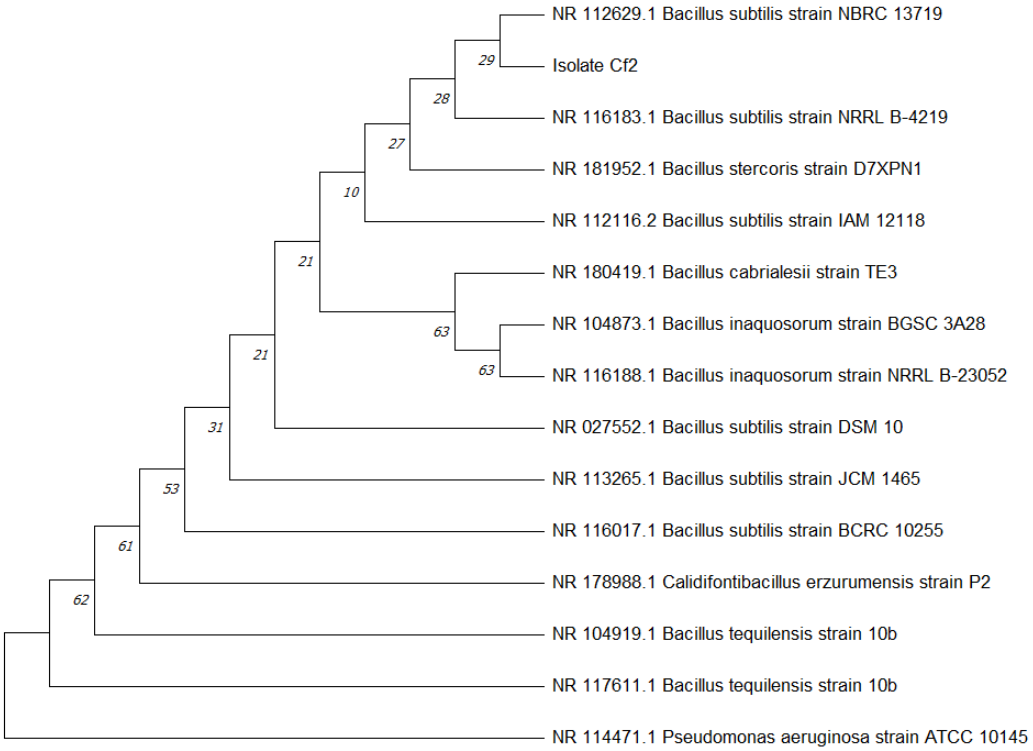

Figure 1. Phylogenetic tree of isolate Cf2 with closely related species

Bacillus species are ubiquitous in nature, inhabiting diverse environments, including freshwater, marine ecosystems, soil, sediment, plants, animals, air, and even human systems. These species also have significant potential for application in agriculture, medicine, and industry (Maughan & Van der Auwera, 2011; Xu et al., 2023). One notable use of *Bacillus* species is as a biodegradation agent. According to Singh & Singh (2017), these bacteria can effectively degrade a range of environmental pollutants, such as pesticides, explosive waste, dyes, and polycyclic aromatic hydrocarbons (PAHs). Also, several bacteria from the genus *Bacillus* have been identified as capable of digesting glyphosate (Badani et al., 2023; Ibrahim et al., 2023; Rusnam et al., 2023).

Among the *Bacillus* species, *B. subtilis* is recognized for its potential to degrade pollutants. Some researchers have reported that these bacteria, isolated from various environments, can degrade glyphosate. This study is not the first to report the glyphosate-degrading capabilities of *B. subtilis*. Yu et al. (2015) have reported that *B. subtilis* strain Bs-15, previously known for promoting plant growth and controlling plant diseases, significantly enhances glyphosate degradation in soil and plays an important role in the bioremediation of glyphosate-contaminated soil. This strain was reported as one of the best glyphosate degraders, with a maximum tolerated concentration reaching as high as 40,000 mg/L (Manogaran et al., 2017). Additionally, Manogaran et al. (2017) proposed a mathematical model to describe the glyphosate degradation rate of *B. subtilis*. Meanwhile, Rusnam et al. (2023) isolated a glyphosate-degrading bacteria from soil near Lake Maninjau, which is likely *B. subtilis*. Isolate Cf2, identified as *B. subtilis* in this study, was previously reported to tolerate 50 ppm glyphosate (Nikmah & Lisdiana, 2024). Although the preliminary studies indicate a lower

tolerance rate, isolate Cf2 is an indigenous bacterium, which may be better adapted for implementation on local farms in Indonesia. Thus, further studies on this isolate are needed to explore its degradation pathways and enhance its degradation capabilities.

The ability of bacteria to degrade glyphosate is closely related to its genetic properties. Generally, microorganisms can degrade glyphosate through two metabolic pathways, namely AMPA (aminomethylphosphonic acid) production and C-P lyase (Feng et al., 2020; Sun et al., 2019). The presence of specific enzymes, which can be predicted by identifying their coding genes in the genome, allows these pathways to occur.

In the first pathway, glyphosate is utilized as a carbon source, which leads to the production of AMPA. This process involves the glyphosate oxidoreductase (GOX) gene. GOX cleaves the C-N bond of glyphosate in the presence of magnesium and flavin adenine dinucleotide (FAD), forming AMPA and glyoxylate. Glyoxylate then enters the Krebs cycle, metabolizing it into its intermediates (Firdous et al., 2020).

The second pathway utilizes glyphosate as a phosphorus source, producing sarcosine and glycine through C-P lyase. This C-P lyase pathway is the most common route microorganisms use for phosphonate utilization. C-P lyase complex has 14 genes that collectively catalyze the breakdown of phosphonates. In this complex, the *phnCDE* genes are known to encode an ATP-binding cassette transporter, *phnF* serves as a repressor protein, and *phnNOP* participates in regulatory or auxiliary roles. The core components of the C-P lyase pathway are represented by the genes *phnGHIJKLM*, with *phnJ* specifically catalyzing the primary C-P cleavage reaction. This reaction converts 5-phosphoribosyl-1-phosphonate to 5-phosphoribosyl-1,2-cyclic phosphate, facilitated by S-adenosyl-l-methionine (Firdous et al., 2020).

The AMPA pathway is the primary route for glyphosate degradation in soil by several Gram-positive and Gram-negative bacteria. *B. subtilis* has also been shown to metabolize glyphosate into AMPA and glyoxylate. Nevertheless, it uses a reaction mechanism different from GOX. Instead of GOX, *B. subtilis* employs glycine oxidase (GO), which catalyzes the deaminative oxidation of glyphosate, resulting in the production of glyoxylate, AMPA, and hydrogen peroxide, with the consumption of 1 mol of dioxygen for each mol of herbicide (Pollegioni et al., 2011).

Based on the identification results of isolate Cf2, which confirmed it as *B. subtilis*, it is likely that this isolate also degrades glyphosate through the AMPA metabolism pathway involving the GO gene. However, further study is needed to validate this predicted degradation pathway and determine the degradation rate of this isolate in order to gain better insight for future application in the bioremediation of glyphosate-contaminated fields.

CONCLUSION

Isolate Cf2 obtained from the rhizosphere in a local chili pepper plantation is identified as Bacillus subtilis based on the similarity of the 16s rRNA gene sequence. This finding provides a foundation for using isolate Cf2 as a promising agent in bioremediation strategies in glyphosate-contaminated environments.

RECOMMENDATION

Additional confirmation using a polyphasic approach is needed to validate the species of isolate Cf2. In addition, further research is required to determine the glyphosate degradation rate of isolate Cf2, independently or in consortium with other bacteria, to provide deeper insight into its potential and inform future application purposes.

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