

Glyphosate Bioremediation of Contaminated Fish-Pond Water by *Paenibacillus* Sp. FUJX 401 from Industrial Activated Sludge

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Abstract. Six glyphosate (GP)-degrading strains were isolated from industrial activated sludge. Among them, strain FUJX 401 showed the highest GP degradability, and was identified as *Paenibacillus* sp. based on morphological observation, physiological biochemical characteristics, and 16S rRNA gene-sequence analysis. *Paenibacillus* sp. FUJX 401 degraded 86.82% GP in minimal salt medium liquid culture medium containing 500 mg/L GP for 7 days. Further bioremediation experiments in fish-pond water containing 200 mg/L GP under laboratory conditions showed that FUJX 401 degraded 95.76% GP after incubation at 37 °C and 150 rpm for 3 days. This study revealed that *Paenibacillus* sp. FUJX 401 had high GP degradability and thus had potential use for the bioremediation of GP-contaminated water.

Introduction

Glyphosate [N- (phosphonomethyl) glycine; GP] is a nonselective, broad-spectrum organophosphate herbicide that is the most widely used in the world [1, 2]. GP is routinely applied in both agricultural and urban settings for the elimination of annual and perennial weeds [3].

In recent years, the agricultural use of GP has significantly increased because of the introduction of genetically altered GP-resistant crops (such as corn, cotton, and soybeans) through genetic engineering technology [4]. GP is applied to control weeds, and consequently improve crop yield.

However, human health and environmental risks associated with the use of GP have been observed in the last decade [5, 6], suggesting that GP poses a considerable threat to the environment because of its extensive use and toxigenic potential [7, 8]. GP has also been found to be toxic to a range of fungi, actinomycetes, and yeasts. A series of recent results found by Relyea [9-11] has suggested that GP may pose significant direct toxic threat to native amphibian species and induce morphological changes in vertebrate animals [12]. GP can be transferred and accumulated into native fish and bivalve species through bio-absorption and bioaccumulation because of its high polarity and water-solubility [13, 14]. Furthermore, GP is obviously harmful to rats at a low concentration [15, 16]. Therefore, how to efficiently remove GP and its metabolite residues from water resource is very meaningful and necessary.

The main process of GP-elimination in soil is degradation by the enzyme systems of GP-degrading microorganisms [17]. A number of GP-degrading bacteria, including *Enterobacter cloacae* [18], *Burkholderia gladioli* [19], *Bacillus megaterium* [20], and *Pseudomonas* [21], have been isolated from polluted soil that was repeatedly exposed to herbicides. However, studies on the GP-degrading micro-organisms isolated from GP-contaminated water are relatively few.

Fish ponds, which are used for reservoir in the wet-season, irrigation in the dry-season, and aquaculture of fresh-water fishes, play an important role in the aquatic ecosystem among farmlands. Fish-pond water is widely polluted by GP, mostly coming from excessive use of GP in the orchard or the teagarden. However, studies on the renovation of GP-contaminated fish-pond water are relatively few. To the best of our knowledge, this study is the first report about the ability of *Paenibacillus* sp. species on GP biodegradation and their application for bioremediation of the aquatic environment contaminated by GP. This paper aims to isolate, identify, and characterize efficient GP-degrading micro-organisms, and then apply them for bioremediation of GP-contaminated water.

Materials and Methods

Environmental Samples and Chemicals

Water samples were collected from fish ponds located in Qianjin Village, Wuyang Town, Nanchang, Jiangxi Province (116.026504, 28.523508), China (No specific permissions were required for these locations. Obtaining water samples for research is a normal and legal activity in this area. We also confirm that the field studies did not involve endangered or protected species). Industrial activated sludge was obtained from an efficiency tank of Jiangxi Zhengbang Chemical Co., Ltd., a large GP-producing plant in Jiangxi province (Permission was issued from the project technician named Debin Guo of Jiangxi Zhengbang Chemical Co., Ltd., We also confirm that the field studies did not involve endangered or protected species, as well as vertebrate animals, embryos, or tissues). Standard GP with 99.2% purity was obtained from Sigma-Aldrich (Germany), while GP samples with 58% purity were obtained from Jiangxi Zhengbang Chemical Co., Ltd.

Culture Conditions of Isolates

In the GP-degrading test, isolates were grown in minimal salt medium (MSM) (1.5g/L K_2HPO_4 , 0.5g/L KH_2PO_4 , 1.0g/L NaCl, 0.5g/L $MgSO_4 \cdot 7H_2O$, and 0.04g/L $CaCl_2$), with 500mg/L GP as the sole source of carbon. The medium was adjusted to pH 7.0 with 40mmol/L NaOH, and then autoclaved prior to the addition of filter-sterilized GP as the sole carbon source.

Isolation of GP-Degrading Strains

Approximately 5g of activated sludge samples was suspended in 100mL of sterilized MSM supplemented with GP to a final concentration of 100mg/L as the enrichment substrate, and then incubated at 37 °C and 150rpm. After 7 days, 10mL of the culture was transferred to 90mL of fresh MSM containing 200mg/L GP, and then incubated under the same conditions. The process was repeated three times until the final content of GP in the fresh MSM was equal to 500mg/L. Afterward, serially diluted bacteria samples were spread on nutrient agar plates (100mg/LGP and 20g/L Agar were added into mineral salt medium) and fungi samples on Czapek's medium plates (2g/L $NaNO_3$, 0.5g/L KCl, 1g/L K_2HPO_4 , 0.5g/L $MgSO_4 \cdot 7H_2O$, 0.01g/L $FeSO_4$,

20g/L Agar, and 100mg/L GP), and then incubated at 37 °C and 30 °C for 4–5 days, respectively. Colonies that appeared on the nutrient agar plate or Czapek's medium plates were separated. The pure colonies were obtained and further selected as the candidate of GP-degrading strains by high GP concentration (500–3000mg/L) tolerance test.

Identification of the Dominant Strain

The dominant strain was first identified from the GP-degrading strain candidates by cellular morphology, gram staining, physiological, and biochemical characteristics in Bergey's Manual of Bacteriology. To further phylogenetically identify the dominant strain, 16S rRNA gene sequencing of the strain was carried out by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. (Shanghai, China). The sequences were then subjected to blast searches against the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The nucleotide sequences were deposited into the GenBank public database. The most similar sequence of the strain was further aligned. Phylogenetic tree was made by MEGA5.1.

Evaluation of GP Biodegradation Capacity

GP Analytical Method. GP in the culture medium and pond water was analyzed by modified method based on Delmonico et al. [22]. High-performance liquid chromatography (HPLC, Agilent Technologies 1260, USA) was conducted on a HYPERSIL-ODS C18 column (5 µm, 4.6 × 150mm; Thermo, USA) with 0.1% (m/v) phosphoric acid in water/methanol (98: 2, v/v) as the mobile phase at a flow rate of 0.8mL/min and column temperature of room temperature (25 ± 1 °C), and then detected by UV detection (VWD, Agilent, USA) at 240nm.

The growth of bacterial isolates was controlled by the change in optical density at 600nm (OD600).

GP Biodegradation Test. The dominant strain was cultured in 50mL of Luria-Bertani medium (3g/L yeast extract, 10g/L Peptone, 10g/L NaCl, 100mg/L GP). After incubation at 37 °C and 150rpm for 30h, the cells of the dominant strain at the late exponential growth phase were collected by centrifugation at 10000 ×g for 5min. The sediment was then re-suspended in the same volume of MSM as the thallus suspension.

The dominant strain (1%, v/v) was inoculated in MSM with 500mg/L GP at 37 °C and 150rpm. Approximately 1mL of the inoculum was picked out every 24h for the GP depletion assay. MSM without inoculation was used as control. All tests were conducted in triplicate in each experiment. The results of control experiments were used in parallel as abiotic negative controls. Degradation rate (DR) was calculated using the following equation:

$$DR(\%) = \frac{C_0 - C_1}{C_0} \times 100\% \quad (1)$$

C_0 : GP concentration of the control, C_1 : GP concentration of the sample.

Bioremediation of Fish-pond Water with GP-degrading Dominant Strain

The water samples were divided into four groups. To observe the degradation effect of glyphosate, all water samples were added to a final concentration of 200mg/L GP to simulate the severe pollution of the water pond. The experiments were conducted as shown in Table 1. All tests were carried out in triplicate at 37 °C and 150rpm for 3 days. Approximately 2mL of samples were collected from the cultures and filtered by

bacterial filters with pore size 0.22 μm for the assay.

Table 1. Operations of fish-pond water degradation experiments

Samples	A	B	C	D
Inoculum concentration (v/v)	0	1%	0	1%
Sterilization (121 $^{\circ}\text{C}$, 20 min)	+	+	-	-

+: Sterilized;
—: Unsterilized.

Results

Isolation of GP-degrading Bacteria

Six GP-degrading strains capable of utilizing GP as the sole carbon source were isolated from the activated sludge by enrichment culture and dilution plate separation. The degradation experiments demonstrated that these isolates exhibited their variable degradability of GP in MSM with 500mg/L GP, and strain FUJX 401 exhibited the highest degradability of 86.82% for 7 days (Fig. 1). In the GP tolerance tests, the highest GP concentration that FUJX 401 can tolerate was 1500 mg/L (data not shown). Other isolates were not further selected and separated because of their relatively low GP degradability.

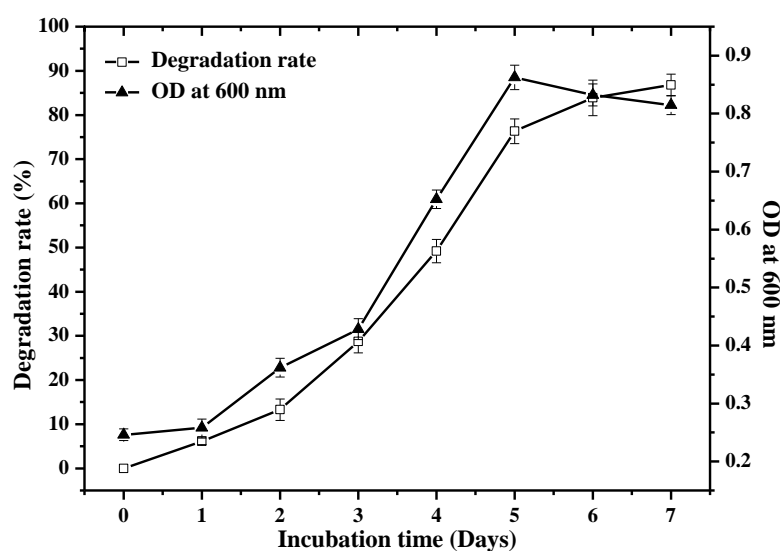


Figure 1. Degradation curve of glyphosate and biomass in minimal salt medium of *Paenibacillus* sp. FUJX 401.

Identification of FUJX 401

In the Luria-Bertani agar plates, FUJX 401 formed entire, flat, smooth, circular, and brownish yellow colonies. The Gram's dye results showed that FUJX 401 was Gram negative and motile with peritrichous flagella. The results of the physiology and biochemistry experiments are shown in Table 2.

Table 2 Physiological and biochemical characteristics of strain FUJX 401

Test items	Results	Test items	Results
Catalase	+	Nitrate reduction	+
Voges-Proskauer test	-	Aerobic growth	+
Milk ring test	+	Citrate used	+
Indole test	-	Glucose with acid production	+
Hydrogen sulfide	-	L-Arabinose	+
Hydrolysis of starch	+	Glycerol	+
Hydrolysis of casein	-	Lactose	+
Hydrolysis of Gelatin	-	D-Mannose	+
Arginine dihydrolase	+	L-Rhamnose	+
Urease	+	Citrate used	+

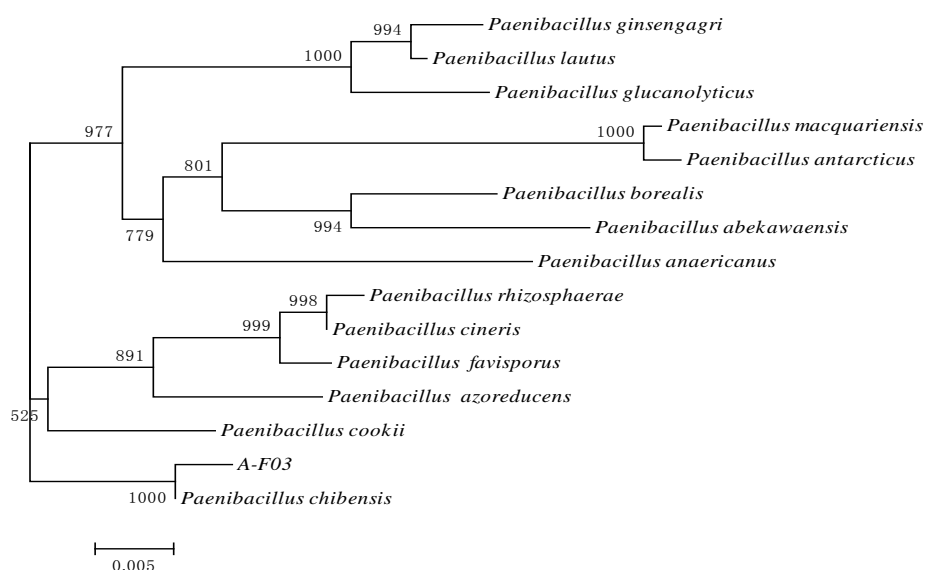
+: Positive result;

-: Negative result.

The 16S rRNA gene sequence (Table. 3) of FUJX 401 was a continuous stretch of 1,477bp and highly (99%) identical to *Paenibacillus* sp.; accession No. JX912706.1 was obtained in NCBI. Subsequently, a phylogenetic tree was constructed using similar 16S rRNA sequences with neighbor-joining methods (Fig. 2). Thus, FUJX 401 was identified as *Paenibacillus* sp.

Table 3 Comparison of the 16S rRNA sequence of strain FUJX 401 with the NCBI strains

Accession No.	Strains	Total score	Identity/%
FN397529.1	<i>Paenibacillus</i> sp. 9-2AIA	2675	99
NR040885.1	<i>Paenibacillus chibensis</i> strain JCM 9005	2662	99
AB681006.1	<i>Paenibacillus chibensis</i> strain NBRC 15958	2660	99
JQ659795.1	<i>Paenibacillus chibensis</i> strain R6-311-1	2651	99
EU497639.1	<i>Paenibacillus</i> sp. Gi-691	2614	99
DQ243814.1	<i>Paenibacillus</i> sp. 2S3	2591	98
FJ432004.1	<i>Paenibacillus chibensis</i> strain ZYb3	2582	99
JN806383.1	<i>Paenibacillus</i> sp. CC-YHH111	2529	98

Figure 2. Phylogenetic tree showing the taxonomic relatedness of strain FUJX 401 with other *Paenibacillus* sp.

Capacity of GP Biodegradation by *Paenibacillus* sp. FUJX 401

The *Paenibacillus* sp. FUJX 401 (1%, v/v) was inoculated in MSM with 500mg/L GP, and then incubated at 37 °C and 150 rpm for 7 days. The GP degradation and biomass curves are shown in Fig. 1. On the first day, an average of 6.24% GP had already been degraded. The mean percentages of GP degradation increased to reach 13.29, 28.71, 49.18, 76.32, 83.88, and 86.82% on the 2nd, 3rd, 4th, 5th, 6th, and 7th day, respectively.

The result in Fig. 1 indicated that *Paenibacillus* sp. FUJX 401 has mighty GP degradability. The growth curve showed that the degradation process mainly occurred from exponential phase, which began in the second day and ended on the fifth day. Afterward, the degradation efficiency increased slowly with the start of the stationary phase. Thus, GP was used as the carbon source of cell component in the growth cycle.

Bioremediation of GP Polluted Fish-pond Water by *Paenibacillus* sp. FUJX 401

The detection results showed that the pH value and chemical oxygen demand (COD) of the water samples were 6.5 and 38.3mg/L, respectively. The degradation efficiency of GP in fish-pond water samples are shown in Fig. 3. After three days of incubation, the degradation efficiency of GP in inoculated sterilized (B) and in nonsterilized (D) fish-pond water samples were 89.35% and 95.75%, respectively. Meanwhile, the degradation efficiency of GP in the control sterilized (A) and nonsterilized (C) fish-pond water samples was only reduced by 12.65% and 18.55%.

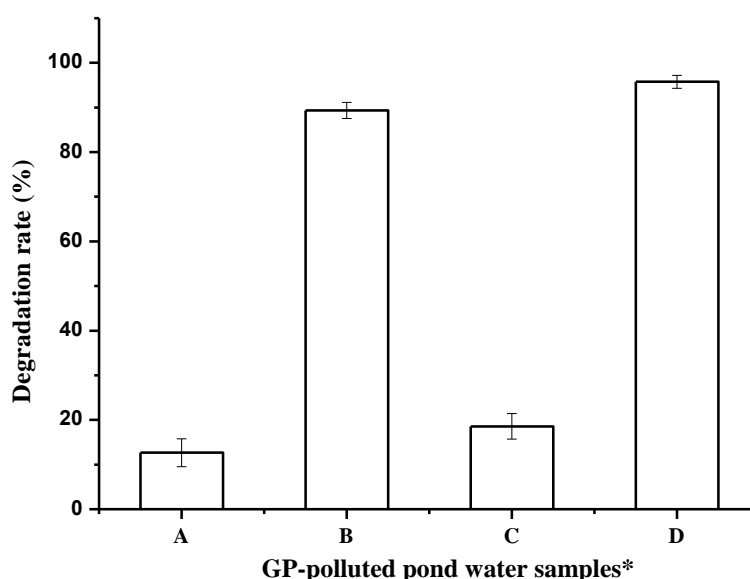


Figure 3. Degradation results of glyphosate in glyphosate -polluted pond water samples by *Paenibacillus* sp. FUJX 401.

Bioremediation experiments in fish-pond water containing 200mg/L GP under laboratory conditions was degraded by three elements: the main part of approximately 76.7% was by *Paenibacillus* sp. FUJX 401, 5.9% by the original micro-organisms in fish pond water, and 12.65% by other factors including illumination and chemical compounds. The biodegradation of *Paenibacillus* sp. FUJX 401 plays the main role in GP degradation.

The results indicated that *Paenibacillus* sp. FUJX 401 can remarkably accelerate GP degradation compared with the original micro-organisms in fish-pond water.

Although other micro-organisms with the ability to biodegrade GP were also found, the *Paenibacillus* species can degrade GP and applied for bioremediation.

Discussion

Currently, three types of treatments are available to degrade GP, including photodegradation, chemical degradation, and biodegradation [8, 18, 23]. Some studies have reported on the GP degradation in water with Advanced Oxidation Technologies. For example, Manassero et al. found that the conversion of glyphosate after 5 h was almost 70% through UV radiation and H₂O₂ treatment [23]. Compared with photodegradation and chemical treatment, biological treatment is the most economical and practical for wastewater treatment^[24]. Many bacteria from soil have been proven for their GP degradability. *Enterobacter cloacae* K7 can degrade 50% of the initial GP concentration (845mg/mL) after 5 days of incubation [18]. *Bacillus megaterium* can also degrade GP, and decreasing the GP concentration from 20mg/mL to 0.80mg/mL [20]. The GP-degradation experiments showed that *Paenibacillus* sp. FUJX 401 isolated from GP-contaminated activated-sludge can degrade more than 80% of 500mg/L GP in MSM within 7 days. Bioremediation experiments of *Paenibacillus* sp. FUJX 401 in the samples of simulated severe pollution pond water with 200mg/L GP under laboratory conditions showed 95.76% GP degradation efficiency after three days of incubation. Compared with *Enterobacter cloacae* K7 [18] and *Bacillus megaterium* [20] from the soil, *Paenibacillus* sp. FUJX 401 has higher GP degradation ability and better adaptability in water environment. This finding indicated that *Paenibacillus* sp. FUJX 401 is a good candidate strain for in situ or on-site bioremediation of the aquatic environment polluted by GP.

Conclusion

A *Paenibacillus* sp. FUJX 401 was been isolated from industrial activated sludge and has been chosen for its potential to degrade glyphosate. In addition, bioremediation experiments in fish-pond water containing 200mg/L glyphosate under laboratory conditions showed that FUJX 401 degraded 95.76% glyphosate. However, the aquatic environment in nature is a complex system, and environmental factors, such as dissolved oxygen and temperature, may affect the degradation of glyphosate by microorganisms. Further studies should focus on the application of *Paenibacillus* sp. FUJX 401 in open wastewater from glyphosate production and glyphosate metabolic pathway of *Paenibacillus* sp. FUJX 401.

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