

Isolation and optimization of a glyphosate-degrading *Rhodococcus soli* G41 for bioremediation

Tuan Ngoc Nguyen (✉ nguyenngoctuan@tdtu.edu.vn)

Ton Duc Thang University <https://orcid.org/0000-0002-1865-6628>

Van Tam Vo

Ton Duc Thang University

Phong The Hong Nguyen

Ton Duc Thang University

Rudolf Kiefer

Ton Duc Thang University

Research Article

Keywords: Glyphosate, *Rhodococcus soli*, biodegradation, bioremediation

Posted Date: November 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1048844/v1>

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Version of Record: A version of this preprint was published at Archives of Microbiology on April 12th, 2022. See the published version at <https://doi.org/10.1007/s00203-022-02875-0>.

Abstract

A widely used herbicide for controlling weeds, glyphosate, is causing environmental pollution. It is necessary to remove it from environment using a cost-effective and eco-friendly method. The aims of this study were to isolate glyphosate-degrading bacteria and to optimize their degradative conditions required for bioremediation. Sixteen bacterial strains were isolated through enrichment and one strain, *Rhodococcus soli* G41, demonstrated a high removal rate of glyphosate than other strains. Response surface methodology was employed G41 strain to optimize distinct environmental factors on glyphosate degradation of G41 strain. The optimal conditions for the maximum glyphosate degradation were found to have the NH_4Cl concentration of 0.663% and glyphosate concentration of 0.115%. Degradation analysis showed 47.1% of glyphosate in soil was degraded by G41 strain after 14 days. The presence of *soxB* gene in G41 strain indicates that the glyphosate is degraded via the eco-friendly sarcosine pathway. The results indicated that G41 strain has the potential to serve as an in-situ candidate for bioremediation of glyphosate polluted environments.

Introduction

Glyphosate (isopropylamine salt of *N*-phosphonomethyl-glycine) is an active ingredient of many organophosphates herbicides (Li et al. 2016; Zhan et al. 2018). Glyphosate has the potential for use in forestry and agriculture to control both grasses and broadleaf weeds. Its activity was demonstrated to inhibit a specific enzyme for the synthesis of a flavonoid (Duke and Powles 2008; Brain and Solomon 2009). However, glyphosate was reported to be persistent for long time in soil. The widespread use of herbicides leads to the accumulation of glyphosate and causes environmental concern because of its toxicity (Mesnage et al. 2015; Nagy et al. 2020). To eliminate glyphosate related health and environmental risks, it is necessary to develop an effective and eco-friendly bioremediation strategy. Glyphosate treatment has been reported via multiple approaches such as adsorption, thermolysis, photodegradation, and biodegradation (Echavia et al. 2009; Manassero et al. 2010). In which, the application of microorganism in glyphosate treatment is considered as the most promising strategy. Some published studies have reported the role of microbes for efficient and rapid bioremediation of glyphosate polluted environments (Ermakova et al. 2010; Yu et al. 2015).

Most of the glyphosate-degrading bacteria belong to the genera *Achromobacter* and *Pseudomonas* (Wackett et al. 1987; McAuliffe et al. 1990; Dick and Quinn 1995; Zhan et al. 2018). Beside, some species of the genera *Alcaligene*, *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Comamonas*, *Geobacillus*, *Flavobacterium*, *Enterobacter*, *Ochrobactrum*, and *Rhizobiaceae* have also been reported (Fan et al. 2012). Most of them was reported to utilize glyphosate as the sole phosphorus source (Obojska et al. 1999; Fan et al. 2012; Yu et al. 2015; Zhan et al. 2018; Firdous et al. 2020). Whereas, the utilization of glyphosate as the sole carbon source was reported in a few bacterial strains including *Achromobacter* sp. LW9, *A. radiobacter* SW9, and *O. intermedium* Sq20 (McAuliffe et al. 1990; Firdous et al. 2018). Interestingly, two strains were reported the ability to use glyphosate as other types of energy source. In which, *C. odontotermis* P2 used glyphosate as the sole carbon and

phosphorus source, whereas *Streptomyces* sp. StC utilized glyphosate as the sole nitrogen and sole phosphorus source (Obojska et al. 1999; Firdous et al. 2020).

Two major degradation pathways have been suggested happening in glyphosate-degrading bacteria (Obojska et al. 1999; Bhaskara and Nagaraja 2006; Echavia et al. 2009; Manassero et al. 2010; Nguyen 2018). The first pathway involves cleavage of the C–N bond by glyphosate oxidoreductase with formation of aminomethylphosphonic acid (AMPA) and glyoxylate. Intermediate metabolite AMPA can be either excreted to the environment because of its bacterial toxicity or further served as a substrate for C-P lyase to produce methylamine and inorganic phosphorus (McAuliffe et al. 1990; Zhan et al. 2018). In the second pathway, glyphosate is converted by C-P lyase into sarcosine and inorganic phosphorus. Sarcosine is then catabolized by sarcosine oxidase to glycine that can be used for protein biosynthesis (Firdous et al. 2018; Zhan et al. 2018). In bacterial strains, the most prevalent glyphosate degradation pathway involves the formation of toxic AMPA and it is negatively regulated by inorganic phosphorus supply. This leads to limitation of using these strains in remediation of glyphosate-contaminated soil because the presence of inorganic phosphorus is commonly detected in soil. The aims of this study were to isolate glyphosate-degrading bacteria and to optimize their degradative conditions required for bioremediation.

Materials And Methods

Bacterial strains and culture conditions

Analytic grade glyphosate was purchased from *Sigma-Aldrich* NY, USA. Enrichment culture broth (g/L) used for the isolation purpose contained the following: **Na₂HPO₄·2H₂O 6.0; K₂HPO₄ 3.0; NH₄Cl 2.0; NaCl 0.5; MgSO₄ 0.2; FeSO₄ 0.01; CaCl₂ 0.02**. The composition of MSB medium has been previously described (Tuan et al. 2011).

Glyphosate-degrading bacteria were isolated from cashew garden soils on which herbicides had been used frequently for more than 10 years in Binh Phuoc province, Vietnam. In the dry season of 2019 (July), surficial soil samples (10 cm depth) were collected from four different locations in an experimental field (11.550°, 106.882°) with 5 cm of diameter steel rings. For the enrichment culture technique, 0.5 g soil was sampled and added to 10 ml of enrichment culture broth containing 0.01% glyphosate. The culture was incubated at 30°C in the dark with shaking at 200 rpm. After 7 days, 10% of the culture was transferred to fresh MSB medium. Glyphosate concentration was then doubled in new medium. After 3 transfers, single colonies with distinct morphology were collected and maintained. All liquid cultures and plates were incubated at 30°C in the dark. Glyphosate-degrading bacteria were grown on medium containing 0.1% glyphosate as the sole carbon source. The effect of various glyphosate concentrations (0.01, 0.05, 0.1, and 0.5%), temperatures (25, 30, 35, and 40°C) on growth was examined for all isolates. Bacterial growth was measured by removing 0.5 ml samples from the culture flask at 600nm with a Jasco model V-730 spectrophotometer. For each test, vials were prepared in triplicate. Medium containing 20% glycerol was used to store bacteria at –80°C.

Identification and phylogenetic analysis

Gram staining was used to determine the type and cell morphology of bacteria under electron microscopy. The 16S rDNA gene of isolates was amplified by PCR using the primers 27F (5'- AGA GTT TGA TCM TGG CTC AG -3') and 1492R (5'- CGG TTA CCT TGT TAC GAC TT -3'). The resulting PCR products were purified with QIAquick spin columns (Qiagen, Valencia, Calif.) and sequenced as described previously (Nguyen 2018). The identification was based on sequence comparisons using BLAST tool from the GenBank database (<http://www.ncbi.nlm.nih.gov>). For constructing a phylogenetic tree, the nucleotide sequences of the 16S rDNA gene from our isolate and the published strains were aligned using Clustal X (version 2.0.3). Phylogenetic stability was supported through Bootstrap analysis with the default value of 1000 trials.

Optimization of parameters for glyphosate degradation

The 3-level-2-factor central composite design (CCD) was applied to evaluate the important operating variables (glyphosate-concentration (X_1) and NH_4Cl (X_2)) for glyphosate degradation. Based on the results of a preliminary experiment (**Supplemental Fig. 1**), the ranges of the variables were chosen as follows: glyphosate dosage is 0.05–0.15% and NH_4Cl is 0.5–0.9%. 13 trials were performed and the independent variables were investigated at three different levels. The response variable (Y) was fitted to a second-order model containing the independent variables as below:

$$Y = \beta_0 + \sum \beta_i X_i + \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

Where Y is the predicted response, X_i and X_j are input variables that influence the response variable Y, β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the linear-by-linear interaction between the X_i and X_j regression coefficients. Minitab (version 16.2.4) was employed for the experimental design.

Assessment of biodegradation and bioremediation

Cultures were grown to the exponential phase in 50 mL volumes with 0.115% glyphosate as the sole carbon and energy source. In order to assess the basal glyphosate utilization rates, the microbial inoculums with final optical density (OD_{600}) of 0.05 were passed to MSB medium containing 0.115% glyphosate. The experimental cultures were performed in triplicate at 30°C in the dark at 200 rpm for 7 days. To determine the abiotic losses of glyphosate, the same MSB medium without any microbial inoculum was also retained. After 0, 1, 2, 3, 4, 5, 6, and 7 days, 1 ml of culture was collected and centrifuged at $6,000 \times g$ for 10 min at room temperature, after that the supernatant was filtered by the 0.22 μm PVDF membrane and used to determine the degradation of glyphosate by ninhydrin reaction as described previously (Bhaskara and Nagaraja 2006) with some modifications. A calibration curve was prepared from a stock solution of 1.5 g/L glyphosate. Aliquots with glyphosate concentration ranging

from 4 to 14 mg/L were transferred to test tubes. Then, 0.5 mL of 5% ninhydrin and 0.5 mL of 5% sodium molybdate were added. The tubes were wrapped in aluminum foil to prevent the light and kept in a water bath at 100°C for 5 minutes. Then the sample was cooled to room temperature and completed with distilled water in 5 mL volumetric tubes. The reading was performed in UV-Visible spectrophotometer at 570 nm and a calibration curve was constructed with the absorbance as a function of glyphosate concentration. For the instrument's baseline, 0.5 mL of ninhydrin and sodium molybdate solution was used, to a total volume of 5 mL. Glyphosate in non-inoculated medium was used as a standard. The analysis was performed in triplicate.

Glyphosate degradation in soil by G41 strain was assayed under artificially controlled conditions as described previously (Yu et al. 2015). For the quantification of glyphosate from soil, soil samples for each treatment were dried in an oven at 65°C for 12 h. 10.0 g of soil was accurately weighted for each treatment and extracted with 25 mL of a solution containing 0.4 M Na₂HPO₄ and 0.4 M Na₃C₆H₅O₇. The solutions were placed in a shaker at room temperature for 24 h. Then, the supernatant was collected by centrifugation at 6000 × g for 30 minutes and used for ninhydrin reaction as above described.

Primer design for detection of target genes

To check the presence of genes involved in glyphosate degradation, the putative glyphosate oxidoreductase (*gox*) and sarcosine oxidase subunit alpha (*soxA*) gene sequences were collected from GenBank database with the top priority from the *Rhodococcus* genus. All sequences of target gene were aligned with Clustal X. In each gene group, the sequences were confirmed to be different by at least 0.5%. To design the PCR primers, the highly conserved regions were selected and used as template. BLAST tool was performed to estimate the specificity of the designed primers (<http://www.ncbi.nlm.nih.gov/blast/>). Target genes were amplified using the G41 genomic DNA as template. The primers designed for amplification of *gox* were F_GOX: 5'-TGG CTS CTH GAY CCR ATG GG-3' and R_GOX: 5'-TCC ATH GGB GTM GCD ATG AA-3' (~ 684 bp). These primers, F_SOX: 5'-CGT CGA TGG ARC ACA TCA AGC G -3' and R_SOX: 5'-TGC TCA TCA TGT TSG TGT AGA -3', were designed for amplification of *soxA* gene (~ 453 bp). PCR conditions used as follows: denaturation at 95°C for 5 min, 30 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 1 min each and final extension at 72°C for 7 min.

Nucleotide sequence accession numbers

The 16S rDNA nucleotide sequence obtained in this study has been assigned the DDBJ/EMBL/GenBank accession number MT830885.1.

Results And Discussion

Isolation and molecular characterization of glyphosate-degrading isolates

In order to understand the degradation of glyphosate in the environment, 16 bacteria were isolated using glyphosate as the sole carbon and energy source (**Supplemental Table 1**). A gram-positive bacterium capable of degrading glyphosate at high efficiency was selected in this study. G41 strain was short, almost round-shaped bacterium. Colonies on nutrient agar medium were *round, slightly convex, smooth, salmon-pink color*, with a diameter of 3 mm within a day at 30°C. G41 strain revealed the shortest generation time and highest optical density in 5 mL MSB medium containing 0.1% glyphosate at 30°C and shaken at 200 rpm (**Supplemental Table 1**). The effect of temperatures, pH, and glyphosate concentrations on growth were also tested and revealed G41 strain exhibited maximum growth at 30°C, pH7.0, and 0.1% glyphosate (**Supplemental Fig. 1**).

In order to identify G41 strain, its 16S rDNA gene was amplified and sequenced. The almost complete 16S rDNA gene sequence of isolated G41 (1348 nucleotides) was determined and its closest relative (99.42%) was the sequences of *Rhodococcus soli* DSD51W (AB847908.1). The phylogenetic tree was constructed using the 16S rDNA gene sequences from our glyphosate-degrading bacterium and type strains of the *Rhodococcus* genus (**Figure 1**). Phylogeny consisted of fifty-five strains and classified into 8 clades. G41 strain located in clade 1 with the presence of *R. agglutinans*, *R. antrifimi*, *R. defluvii*, *R. kunmingensis*, *R. subtropicus*, *R. equi*, and *R. soli*. The phylogenetic analysis indicated that G41 strain was attached with *R. soli* DSD51W with bootstrap value of 99% (**Figure 1**). Considering the results, the isolated G41 strain was identified as *R. soli* G41. The most commonly isolated glyphosate-degrading bacteria belong to the genera *Achromobacter* and *Pseudomonas* (Wackett et al. 1987; McAuliffe et al. 1990; Dick and Quinn 1995; Zhan et al. 2018). Beside, some species of the genera, *Alcaligene*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Comamonas*, *Geobacillus*, *Flavobacterium*, *Enterobacter* and *Rhizobiaceae* have been reported for glyphosate degradation (Obojska et al. 1999; Fan et al. 2012; Yu et al. 2015; Zhan et al. 2018; Firdous et al. 2020). However, the isolation of the *Rhodococcus* genus as the glyphosate utilizing bacteria has not been reported so far. Therefore, this finding adds to the known list of glyphosate degrading bacteria that can be used for decontaminating glyphosate polluted environment.

Optimization of glyphosate degrading conditions by *Rhodococcus soli* G41 strain

In this study, the interaction of two important factors (the dosage of glyphosate and NH₄Cl content) on glyphosate degradation was studied by 3-level-2-factor CCD analysis and response surface methodology. Experiments were performed to evaluate the combined effect of two important parameters on glyphosate degradation using bacterial isolate of *R. soli* G41 strain. The observed and predicted generation time (hours) were shown in **Table 1**. The fitting polynomial equation (2) was calculated after data fitting.

$$Y = 14.4644 - 0.5437X_1 + 0.3399X_2 + 1.4965X_1^2 + 1.6796X_2^2 + 0.5807X_1X_2 \quad (2)$$

The efficacy of model was determined by the coefficient ($R^2 = 0.985$) which demonstrated that independent variables ascribe 98.5% variability to glyphosate degradation. High value of the adjusted determination coefficient (Adj. $R^2 = 0.9743$) also showed the best fit of the model. The analysis of variance illustrated that the fitted model was statistically valid with high model F-value (91.48) and low p values ($p < 0.001$). The three-dimensional response surface plot was shown in **Fig. 2**. The glyphosate dosage ($p < 0.001$) had a greater influence on glyphosate degradation than the NH_4Cl dosage ($p = 0.003$). According to the regression model, the shortest generation time of 14.3846 (hours) was obtained under the following conditions: $X_1 = 0.115\%$ and $X_2 = 0.663$ g/L. The generation time under the optimized condition was observed at 14.25 ± 0.38 (hours), which was in close agreement with the predicted model. Thus, it is believed that the model is reliable for describing the effects of glyphosate and NH_4Cl dosages on glyphosate degradation.

Biodegradation of glyphosate by *Rhodococcus soli* G41 strain

The growth and different glyphosate degradation rates were observed for G41 strain, whereas *E. coli* and *B. subtilis* were unable to grow. It was showed that an increase in turbidity is directly proportional to glyphosate degradation (**Fig. 3A**). G41 strain degraded glyphosate at 1150 mg/L initial concentration in MSB medium. After 7 days of incubation, 42.7% of initial amount of glyphosate was degraded and an increase in cell mass was observed. Only few bacterial strains have been reported to use glyphosate as the sole carbon and energy source. In which, *A. radiobacter* SW9 and *Achromobacter* sp. LW9 were isolated from a bench scale sequencing batch reactor degrading a waste stream containing glyphosate (McAuliffe et al. 1990). Strain LW9 and SW9 converted glyphosate at 1099 mg/L into AMPA with a small amount of degradation of AMPA (McAuliffe et al. 1990). *O. intermedium* Sq20 was able to degrade glyphosate at 500 mg/L initial concentration in minimal salts medium within four days at ambient temperature and neutral pH (Firdous et al. 2018). In 2020, Firdous et al. described that *C. odontotermis* P2 was capable to utilize glyphosate as carbon and phosphorus source (Firdous et al. 2020). The strain P2 degraded glyphosate at 1500 mg/L under the optimal conditions and resulted in a maximum degradation of 90% within 104 h. Thus, a good agreement among the isolated bacteria was observed concerning the glyphosate degradation. It indicates that isolated G41 might serve as a potential candidate for glyphosate bioremediation.

Soil bioremediation

The efficiency of glyphosate bioremediation by the introduced *R. soli* G41 was estimated under the controlled conditions in laboratory-scale experiments. The determination of glyphosate was performed in two types of samples: Glyphosate soil sample (soil with glyphosate irrigation at day 0, 7, and 14), and

glyphosate + bacteria soil sample (soil with both glyphosate irrigation and *R. soli* G41 supply at day 7 and 14). The difference in glyphosate content between glyphosate soil sample (day 0) and glyphosate soil sample (day 7) was 8.9%, whereas between glyphosate soil sample (day 0) and glyphosate soil sample (day 14) was 10.7% in soil with only indigenous microorganism (**Fig. 3B**). The result was consistent with the earlier studies in which the maximum value of glyphosate biodegradation did not exceed 11% in soil quantitatively assessed only for indigenous microorganisms with different levels of adaptation to glyphosate. The difference in glyphosate content between glyphosate soil sample (day 0) and glyphosate + bacteria soil sample (day 7) was 42.2%, whereas between glyphosate soil sample (day 0) and glyphosate + bacteria soil sample (day 14) was 47.1% (**Fig. 3B**). The difference between treatments (33.3% at day 7 and 36.4% at day 14) suggests an active role of *R. soli* G41 in decreasing the amount of glyphosate in soil. In previous studies, bioremediation of glyphosate-contaminated soil was investigated by the introduced strains. In which, the degradation of glyphosate by the introduced *Achromobacter* sp. Kg16 and *O. anthropi* GPK3 decreased 45.8% and 29.0%, respectively after 21 days of treatment (Ermakova et al. 2010). The treatment with *B. subtilis* Bs-15 showed 71.57% reduction in glyphosate concentration in unsterilized soil (Yu et al. 2015). Thus, results in bioremediation of glyphosate-contaminated soil by the introduced G41 also indicated that there is great potential for intensifying the process of soil cleanup after weed treatment to prevent the accumulation of glyphosate in soil and the elimination of soil toxicity.

Proposed mechanism for glyphosate degradation by isolated bacterium

Two major pathways of glyphosate biodegradation have been reported (Obojska et al. 1999; Bhaskara and Nagaraja 2006; Echavia et al. 2009; Manassero et al. 2010; Nguyen 2018). One pathway involves glyphosate catabolism resulting in glyoxylate and AMPA production by GOX in the presence of magnesium and flavin adenine dinucleotide. AMPA acute toxicity has been described for rats, fish, and aquatic invertebrates (Mesnage et al. 2015; Nagy et al. 2020). *Achromobacter* sp. LW9 and *A. radiobacter* SW9 were reported to utilize glyphosate as a sole carbon source in presence of phosphate to produce AMPA (McAuliffe et al. 1990). Although many efforts have been done to detect the presence of *gox* gene in G41 strain but it is undetectable. In addition, there is no data available in literature about *gox* identification in the genome from the *Rhodococcus* genus. These findings indicate the absence of the AMPA pathway for glyphosate degradation in G41 strain. The second pathway catalyzes glyphosate to produce sarcosine and phosphate via C-P lyase. Sarcosine is further processed to glycine via SOX. Glycine can be used for protein and nucleic acid biosynthesis (Firdous et al. 2018; Zhan et al. 2018). To date, the C-P lyase with high-specificity to glyphosate has not been clearly characterized at genetic and biochemistry level. In this study, a partial *soxA* gene sequence (453 bp) was successfully amplified and further confirmed by sequencing from *R. soli* G41 (**Supplemental Fig. 2**). BLAST analysis of *soxA* sequence obtained from G41 strain revealed 98.9% identity with *soxA* gene of *R. opacus* (AP011115.1). The result indicated the presence of the sarcosine oxidase pathway in G41 strain. Unlike

the AMPA pathway, the sarcosine oxidase pathway is more eco-friendly and has been reported in *O. intermedium* Sq20 that utilized glyphosate as sole carbon source in presence of phosphate (Firdous et al. 2018).

Conclusion

In this study, the isolation and identification of *Rhodococcus soli* G41 on the basis its potential to metabolize glyphosate were performed. This work is a new addition to the list of already reported glyphosate degrading bacteria. Identification of potential *sox* gene indicated the presence of the sarcosine oxidase pathway in *R. soli* G41. Moreover, response surface methodology provided imperative information about optimization of two important growth parameters and their mutual effects for maximum degradation of glyphosate. Capability of *R. soli* G41 to degrade glyphosate demonstrated that it would be a good candidate for decontamination of glyphosate affected environment.

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Tables

Table 1. Central composite design for optimization of the degradative parameters of glyphosate with G41 strain

Run	Factor				Generation time (hours)	
	Glyphosate		NH ₄ Cl		Actual value	Predicted value
	X1	A (%)	X2	B (%)		
1	-1	0.05	-1	0.5	16.3215	16.4985
2	1	0.15	-1	0.5	15.1532	15.1432
3	-1	0.05	1	0.9	16.2660	16.3965
4	1	0.15	1	0.9	16.2660	16.2095
5	-α	0.0295	0	0.7	16.6978	16.5046
6	+α	0.1705	0	0.7	15.3452	15.4172
7	0	0.1	-α	0.418	15.8976	15.8040
8	0	0.1	+α	0.982	16.5115	16.4839
9	0	0.1	0	0.7	14.3631	14.4644
10	0	0.1	0	0.7	14.3264	14.4644
11	0	0.1	0	0.7	14.5473	14.4644
12	0	0.1	0	0.7	14.5803	14.4644
13	0	0.1	0	0.7	14.5034	14.4644

Figures

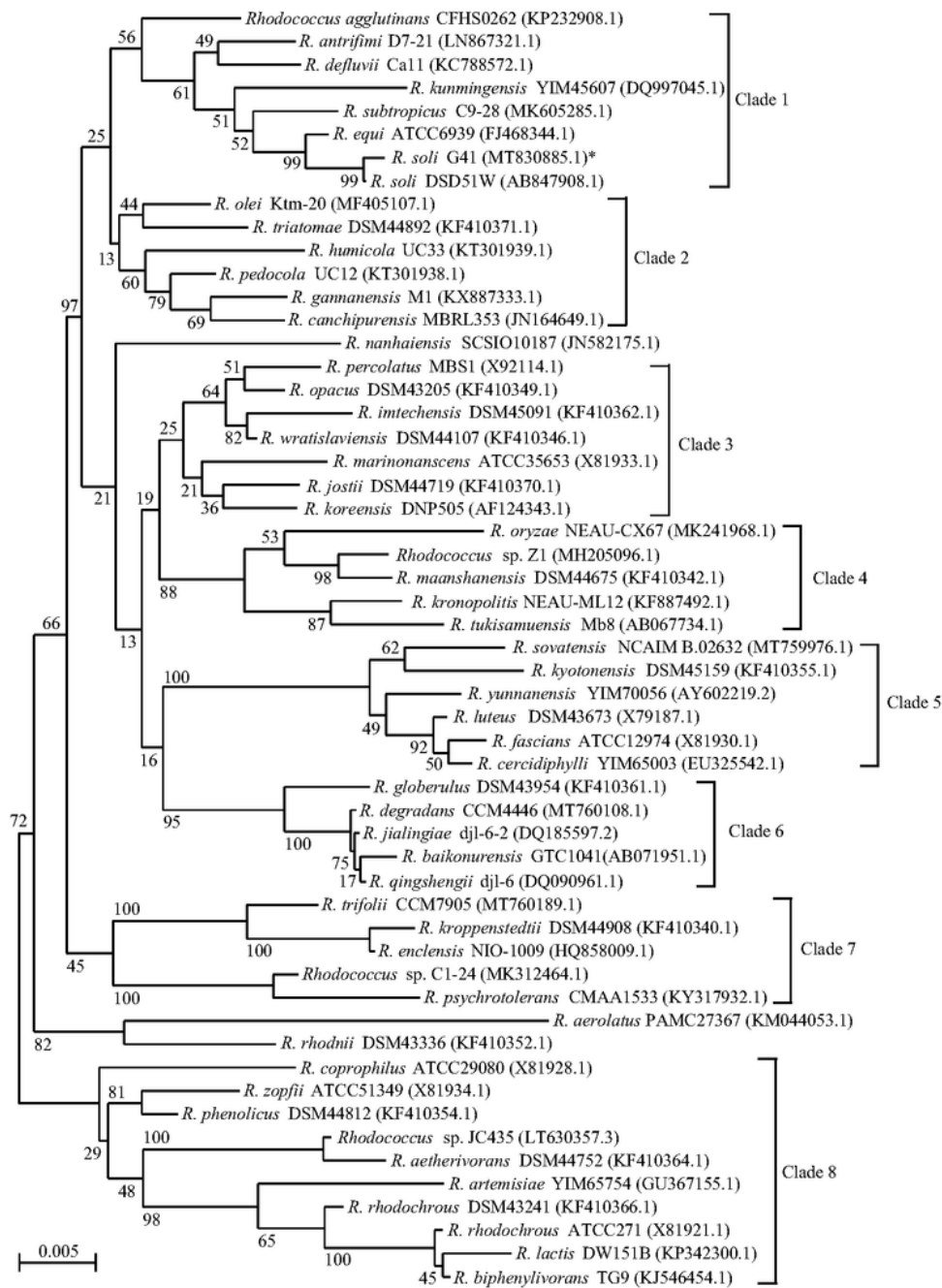


Figure 1

Phylogenetic neighbor-joining tree based on the nucleotide sequences of 16S rDNA genes of locally isolated (indicated by star) and type bacterial strains of the genus *Rhodococcus*. The strains are indicated by their EMBL/GenBank/DDBJ accession number followed by their species names. Bootstrap values, indicated at the nodes, were obtained from 1000 bootstrap replicates and are shown as percentages. Bar indicates 0.5% sequence divergence.

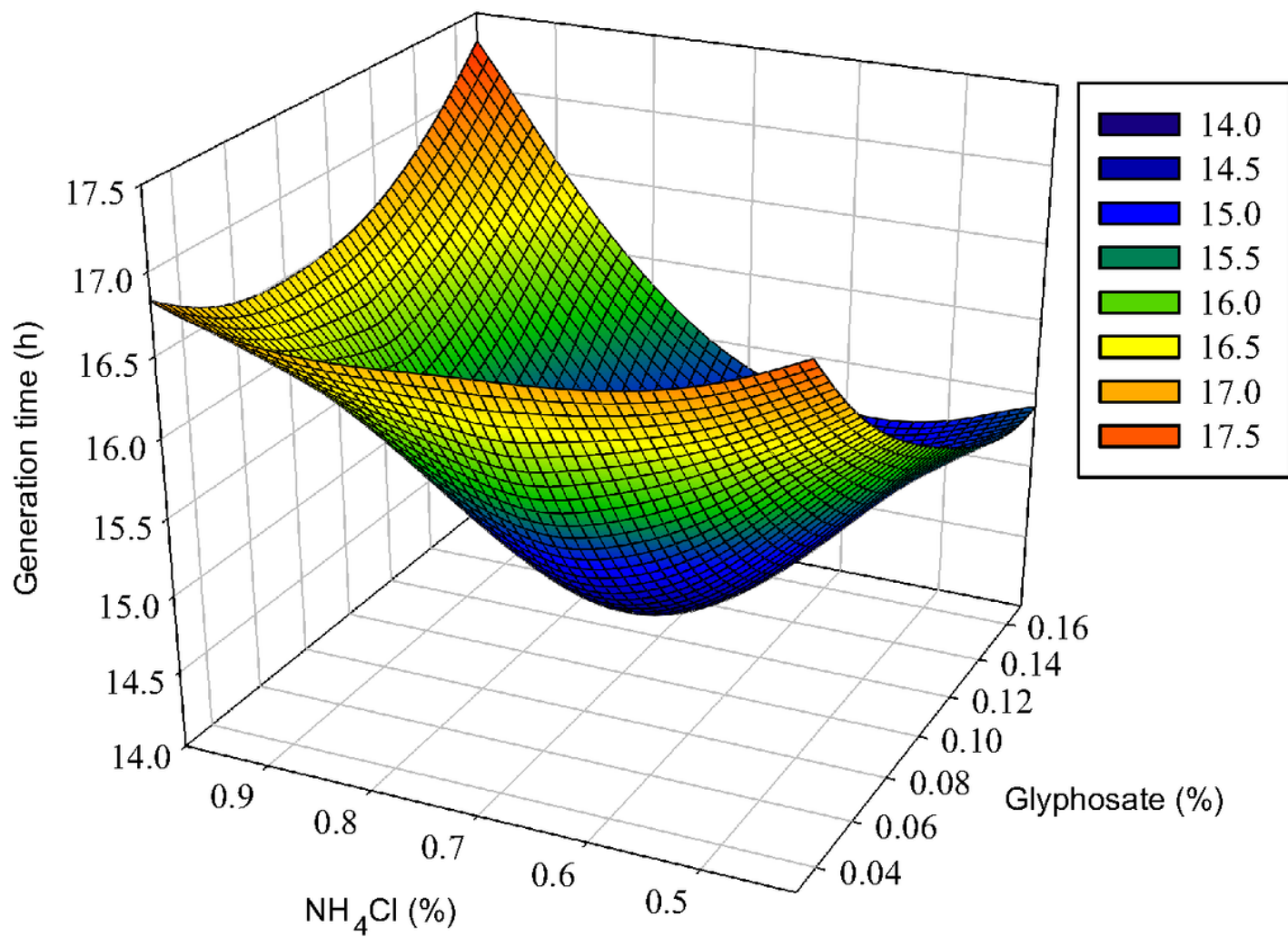


Figure 2

Response surface plot for generation time by *Rhodococcus soli* G41 versus NH₄Cl and glyphosate concentration.

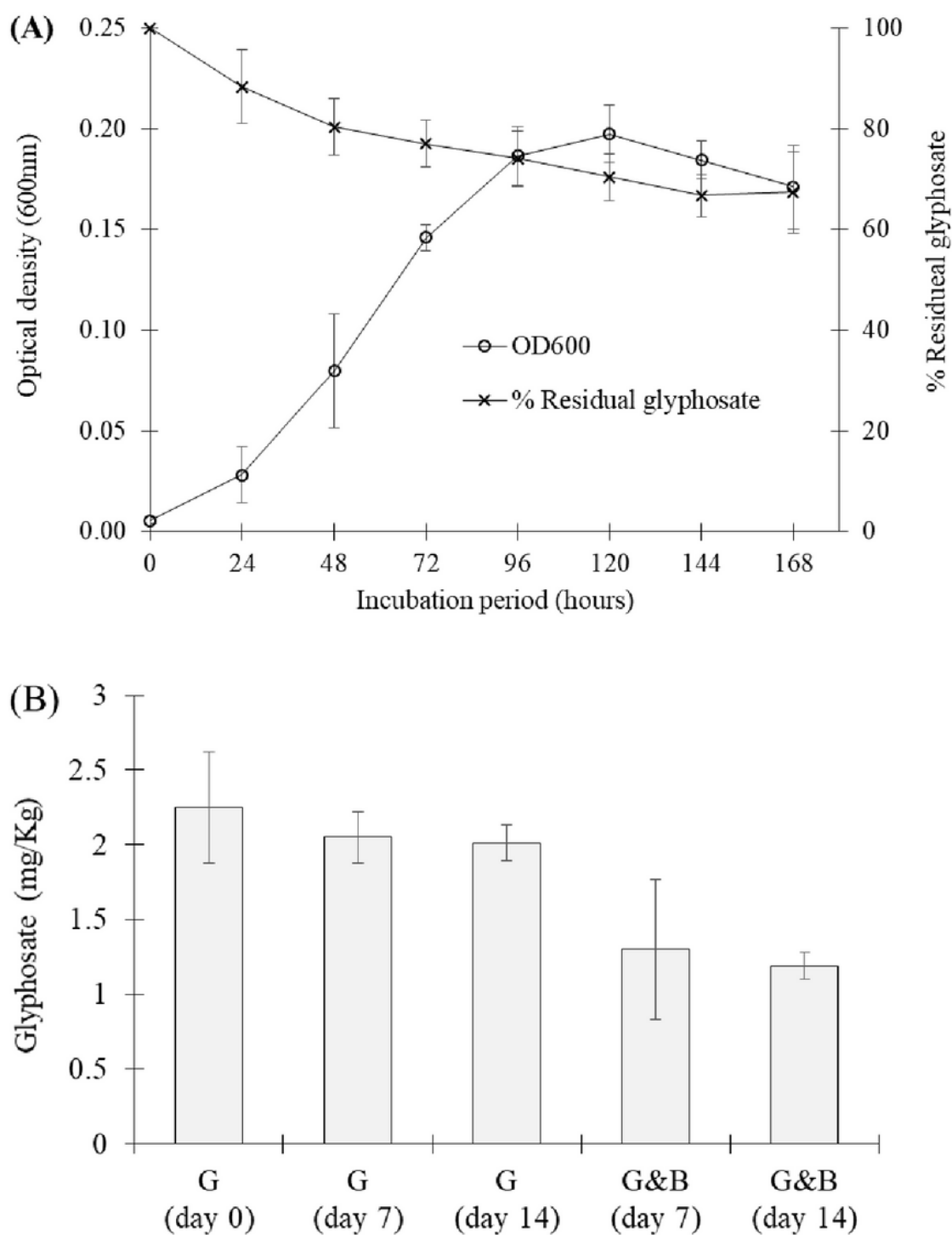


Figure 3

(A) Bacterial growth and glyphosate removal by bacterium *Rhodococcus soli* G41 in liquid cultures. The microbial inoculums with final optical density (OD600) of 0.05 were passed to MSB + 0.115% glyphosate medium. The experimental cultures were performed at 30°C in the dark at 200 rpm for 7 days. (B) Determination of glyphosate concentration for different treatments. Glyphosate was determined by the

standard addition method at days 0, 7, and 14 for glyphosate (G) and glyphosate + bacteria (G&B) treatments. Data are expressed as the mean \pm error of three independent experiments.

Supplementary Files

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