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In-vitro **biodegradation of Glyphosate using genetically improved bacterial isolates from highly polluted wastewater**

Nivien A. Abosereh[a], Rasha G. Salim[a]*, Ahmed F. El-Sayed[a], Maher A. Hammad[b] and Ghada M. Elsayed[a]

[a] Department of Microbial Genetics, National Research Centre, Dokki, Giza, Egypt.
[b] Department of Plant Protection, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Abstract

Due to the problems assigned by unregulated and indiscriminate applications of pesticides, adverse effects to human health, different life forms, and ecosystems were evolved. Development of technologies that guarantee their elimination in a safe, efficient, and economical way is important, among these strategies, bioremediation that overcomes the limitations of traditional methods for the disposal of hazardous compounds. An organophosphorus pesticide, Glyphosate (N-phosphono methyl glycine), was the most persistent pesticide in the Al-Jabal Al-Asfar drain water canal. Four bacterial isolates recorded a notable degradation behavior toward glyphosate with various capabilities. The PCR amplification of the 16s rDNA gene was employed to identify these bacterial isolates. They were identified as *Bacillus cereus* NRC1-PP, *Pseudomonas alcaligenes* NRC2-Gly, *Pseudomonas stutzeri* NRC3-8PS, and *Bacillus licheniformis* NRC4-1BL and deposited at GenBank. *Bacillus cereus* NRC1-PP, as the highest degrader, biodegraded 28.96% of Glyphosate when injected in minimal salt media after ten days. Enhancement of Glyphosate biodegradation potential for *Bacillus cereus* NRC1-PP through physical mutagen UV radiation and chemical mutagen Ethyl methane sulfonate (EMS) was implemented. The biodegradation fitness was increased to 2.5-fold in UV-10 bacterial mutant. Protein profiles of *Bacillus cereus* NRC1-PP and its mutants were investigated by SDS-PAGE. Dendrogram of SDS-PAGE based on unweighted pair group method with arithmetic averages algorithm (UPGMA) divided and categorized into 2 main clusters according to similarity coefficient. The enzymatically- generated degradation products of Glyphosate by GC/MS were detected. The treated samples were presented 12 metabolites were detected in the case of UV-10 treatment; however, only presented 7 metabolites were assigned in the untreated sample (control). These metabolites included amino methyl phosphonic, and new ions such as C_2N_2O and $C_3H_4O_3P$. The results of this study indicate that bacterial isolate and their mutants are good candidates for Glyphosate biodegradation in safe and efficient behavior.

Keywords: wastewater; Glyphosate; bioremediation; 16srDNA; mutation; SDS-PAGE

1. Introduction

Despite the use of pesticides highlights the obvious benefits of crops, the intensive and widespread use of pesticides by contaminated water resources in many countries raises serious environmental and health concerns, bioremediation has been recommended as a suitable alternative for dealing with pesticides-related, where various microorganisms have great potential for the biodegradation of various pesticides, organophosphate pesticides constitute for more than 40% of all pesticides used in agriculture, and underdeveloped countries concern agricultural poisoning due to the lack of monitoring procedures [1]. An example of Organophosphate pesticides, Glyphosate is considered a broad-spectrum used to control weeds in agricultural regions across the world. Unfortunately, Glyphosate was approved by many reports as a carcinogenic compound causing organ failure and inhibition of the mitochondrial succinate dehydrogenase activity [2,3], genotoxicity, and cutaneous toxicity [4,5], For this reason, developed approaches are considered urgent to manage Glyphosate degradation, for example, the microbial degradation [6] a significant role by microorganisms in this issue was evolved to remove the pollutants reported in sediments, water, and soil; regardless of remediation methods [7]. Degradation of Glyphosate can be achieved using different mechanisms such as (absorption, photolysis, thermolysis, and biodegradation with catabolic enzymes), But Glyphosate is highly resistant to chemical degradation and photodecomposition, whereas the half-life of GP in the soil varies from 2

*Corresponding author e-mail: rasha_gomma@yahoo.com.; (Rasha G. Salim).
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to 197 days, depending on the soil type, the level of microbial activity, and climate conditions. Another promising option for reducing the environmental and health problems provided by Glyphosate and its residues is to use an ecologically friendly strategy such as bioremediation. Another issue that is considered is the metabolites and their toxicity resulting from microbial degradation of Glyphosate; Therefore, the GLY metabolites evolved by microbial degraders have become essential to study. In this concept, various studies have reported microbial capacity as an effective tool in bioremediation.

Different authors have reported the degradation of Glyphosate through different microorganisms such as bacteria for example, *Achromobacter sp.* Kg 16 and *Ochrobactrum anthropic* GPK 3 [8] *Bacillus cereus*CB4 [9], *Enterobacter cloacae*, *Enterobacter sp*, *Pseudomonas flurescens* [10], *Bacillus aryabhattai* FACU [11], Actinomycetes, and fungi, that have been isolated from sites already treated by the pesticides [12-14]. These microorganisms almost use Glyphosate as a source of phosphate, carbon, or nitrogen during metabolism to produce an amino methyl phosphonic acid (AMPA) and have been identified and characterized [15]. Molecular identification of these bacterial isolates by 16S ribosomal RNA (rRNA) genes have been the most predominantly barcoding gene for bacterial classification [16].The bioinformatics analysis was applied to reservoirs containing herbicide-contaminated wastewater to identify critical key genes, which functions were relevant for survival in these conditions, by performing only 16S rDNA amplicon sequencing, and analyzing the genes present in the identified OTUs [17]. Mutation techniques have been used to improve glyphosate degradation bacteria. Physical and chemical mutagens have been applied by many researchers to generate new biotypes [18]. Ultraviolet light (UV), ionizing radiation, and chemicals (as EMS, nitrous acid, hydroxylamine, and transposons) can randomly induce modifications in organism's genome [19]. A Protein banding pattern using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique was employed to discriminate between the wild type and its mutant. It has been used to evaluate the genetic diversity among different species of bacteria [20]. The present study aimed to investigate the bioremediation of GP-contaminated wastewater by the isolated bacterial strains able to degrade Glyphosate and improve their potential by mutation induction.

Material and methods

2.1Chemicals and reagent

The N-(phosphonomethyl) glycine, isopropyl ammonium salt of glyphosate known as Roundup (containing 480 g active ingredient/L of glyphosate, ROTAM- Hong Kong) was purchased from a local store supplying agricultural products in Egypt. For the isolation of bacteria using glyphosate as the sole source of carbon, mineral salt medium (MSM) was used. The composition of the medium in g/L of distilled water, pH (7.0) was KH_2PO4 (1.5), NaCl (0.5), Na_2HPO_4 (0.6),NH_4SO_4 (2), $MgSO_4.7H_2O$ (0.2), $CaCl_2$ (0.01) and $FeSO_4.7H_2O$ (0.001)[12]. Supplemented with 300μg/L Glyphosate and was used to enrich and isolate Glyphosate-degrading strains

2.2 Sampling

Water samples were collected from three different locations of Al-Jabal Al-Asfar drain water canal these sampling sites were selected according to1km distance between them in Qaliubiya Governorate, Egypt Figure 1. Water samples were collected for a year (one time every three months) starting in April 2020 to February 2021 representing Spring, Summer, Autumn, Winter, and Fall of 2021. Samples of 3L each were collected from a mid-point of Al-Jabal Al-Asfar drain water canal in cleaned, sterilized and solvent washed glass brown bottles at 50 cm below the water surface. The bottles were labeled, transported within 3 hrs of collection to the laboratory in ice container samples had filtered through fiberglass filter to remove turbidity and debris; and stored at 4°C prior to extraction. Then analyzed for water pesticide residues pollutants. And samples had used for isolation of different potential bacterial strains in pesticide degradation**.**

Figure (1): locations of the sampling sites from of Al-Jabal Al-Asfar drain water canal.

2.3 Monitoring of pesticides residues in wastewater samples

Wastewater samples were collected in sterile glass bottles that were transferred to Central Agriculture Pesticides Lab (CAPL), Agriculture Research Center, Giza, Egypt to determine pesticides residues using LC-MS/MS (ABSCEX 6500 Q-TRAP) system.

2.4 Bacterial Isolation from wastewater and sediment

Glyphosate-degrading bacteria were enriching and isolating as follow: approximately 1 mL of each water sample was added to 49 mL of MSM in a 100 mL conical flask containing 300 µg / L Glyphosate (Roundup) and soaked for seven days in the dark at room temperature (37 ° C) with stirring. shake at 150 rpm. 5 ml of each suspension was placed in fresh MSM containing 300 µg / L Glyphosate (Roundup) and incubated for another 7 days.

2.5 Evaluation the Glyphosate Biodegradation by the bacterial isolates in liquid minimal salt media:

Glyphosate 300µg/L, as a sole carbon source, was added to minimal salt media to which the bacterial isolates were inoculated at pH 7 in a triplicate manner. The incubation conditions were optimized at 37°C and 150 rpm on an orbital shaker (Thermo fisher scientific, UK) For 10 days. Simultaneously, non-inoculated media as control samples were also run in parallel to the other cultures. Glyphosate residues were determined in Central Agriculture Pesticides Lab (CAPL), Agriculture Research Center, Giza, Egypt. Glyphosate residues were calculated by the following equation

$$\text{Degradation\%} = \frac{\text{(Residual amount of blank control- Residual amount of sample)}}{\text{Residual amount of blank control}}$$

2.6 Molecular identification by 16S rDNA barcoding

Genomic DNA was extracted from the most potent bacterial isolates using the (QIAamp DNA Mini Kit, Qiagen, Germany) according to manual instructions. The polymerase chain reaction was employed to amplify the 16S rDNA gene using DNA as a template in Thermal cycler System 2400 (Perkin-Elmer Norwalk, Connecticut, USA) using the universal primers; 8F forward primer sequence (5'AGAGTTTGATCCTGGCTCAG3') and 1492 R reverse primer sequence (5'GGTTACCTTGTTACGACTT3'). The amplification reaction was carried out in 50 µL reactions by using a PCR master mix (Emerald AmpGT PCR master mix (2x premix), Takara, Japan) using the following program: 94°C for 3 min as the initial denaturation step, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 10 min. QIAquick Gel Extraction Kit (Qiagen, Germany) was used to purify the PCR products. Step of DNA resolving was performed by electrophoresis on 1.5% Agarose gel using a 100bp DNA Ladder to detect the molecular length of the target gene (thermoscientific).The PCR product was subjected to sequencing in a biomedical laboratory of colors (cliniclab, Egypt) via the Sanger sequencing method using a 3500 genetic analyzer, and a big dye x terminator kit (Thermfisher, USA). The obtained sequences were compared with other known sequences found in the GenBank database using the BLAST algorithm of the NCBI database. All sequences were deposited in the GenBank database under accession numbers. The phylogenetic tree was constructed using MEGAx [21] by Performing distance matrix analysis using the neighbor-joining method (NJ).

2.7 Genetic improvement of Glyphosate biodegradation by mutation induction

2.7.1 Ethyl methane sulfonate (EMS) mutation induction:

The following procedures were used to achieve EMS mutation: A single colony of *Bacillus cereus* was inoculated in 20 mL LB and incubated at 30 ° C for 18 h at 120 rpm. In the next morning, 1 mL of bacterial culture was the re-cultured in 20 mL of LB at the same conditions but for 4 h until the optical density reached 0.6 at 600 nm. One ml of bacterial growth was centrifuged for five minutes at 10 rpm, and then the pellet was re-suspended in 5 mL phosphate buffer pH 7 and re-centrifuged. This step was repeated for three times. The pellet was re-suspended in 5 ml of phosphate buffer pH 7, 100 µL of the sample was spread on LB agar plates at optimum growth conditions to determine the CFU/mL and considered as control. According to El-Sayed *et al*[22], different concentration, 100, 200, 300, 400 and 500 µL/mL of EMS (1g/mL, Sigma Aldrich) was added to 1 ml of bacterial suspension in sterilized tubes for 30 min under shaking conditions at 30°C. The reaction was stopped by the addition of 4 mL of 5 % sodium thiosulfate. The pellet was collected, washed, and re-suspended in 1 mL of phosphate buffer. Suitable dilutions of 100 µL from each treatment were spread on LB agar plates at the same conditions as control. Estimation of survival percentages was calculated for each treatment comparison to control after the development of single colonies on growth media. All treatments were replicated by three times.

2.7.2 Ultraviolet mutation induction:

The procedures used to achieve this experiment were the same as used in EMS mutation induction except, the bacterial suspension was exposed to UV source (A 30-w germicidal lamp at 254 nm, VL-130.G, Vilber, Germany) at a distance of 20 cm for three, five, seven, ten and 15 minutes. All plates were

kept in dark to prevent photo repair. Prior to spreading on an LB agar medium, samples were immediately diluted 1:10 in LB medium-containing flasks wrapped in tin foil and grown to saturation. Then, appropriate cell dilutions were inoculated on LB agar plate and incubated at optimum growth conditions in the dark until the development of single colonies.

2.8 Metabolites extraction

The Glyphosate pesticide and its metabolites products were detected in the national research centre using the GC-MS system. The culture medium was extracted after 10 days. The bacterial suspension was centrifuged (5000 xg for 5 min at 4ºC) and the supernatant was stored at −80ºC until sample preparation. The Extraction was performed by adding 1000 µL of methanol: acetonitrile: water (2:3:1) to 100 µL of the specimen. Samples were vortexed for 5 min, sonicated for 5 min, and finally centrifuged at 14 000 xg for 10 min at room temperature. Before GC-MS analysis, the extract was dried with nitrogen and resuspended in 50 µL of BSTFA incubated in a Dry Block Heater at 70 ºC for 30 min.

2.8.1 Gas chromatography–mass spectrometry analysis (GC-MS).

The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, and Cairo, Egypt. The GC was equipped with HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 μm film thickness). Analyses were carried out using Hydrogen as the carrier gas at a flow rate of 1.0 ml/min at a splitless, injection volume of 1 µl and the following temperature program: 50 °C for 1 min; rising at 10 °C /min to 300 °C and held for 20 min. The injector and detector were held at 250 °C. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 30-700 and solvent delay 9 min. The mass temperature was 230°C and Quad 150 °C. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

2.9 Analysis of protein profile using SDS-PAGE fingerprinting of wild type *Bacillus cereus* and its mutants' strains

SDS-PAGE was used to analyze the proteins of wild type and mutants. A loopful of wild type and mutants were implanted at 120 rpm for 3 days at 30ºC in a 100-ml conical flask containing 10 ml of nutrient broth. Bacterial cultures were centrifuged and protein extraction was performed according to Von-Tersch *et a.l* [23]. To compare the protein profiles of the wild strain and their mutations, 12.5 % SDS-PAGE [24] was used to separate the proteins. After size fractionation, the proteins were stained with Coomassie Blue R-250 dye to visualize them. The molecular masses were determined using complete laboratory, version 1.10 software based on pre-stained protein markers purchased from Thermoscientific after the gels were imaged using the Gel Doc 2000 system. The protein profiles were recorded as binary data, that is, 1 or 0 based on the presence or absence of bands. The similarity and relationship between the protein traces of test mutants were represented in a dendrogram using the unweighted pair group method with arithmetic averages algorithm (UPGMA) of whole-cell protein patterns of mutants.

2.10 Statistical analysis

Triplicates of all in vitro studies were carried out. Standard deviations for each experimental result were computed using Microsoft Excel Spreadsheets for statistical analysis. The outcomes have been presented. Three independent determinations are used in this investigation. The standard deviation is shown in the bar score.

3. **Results and discussion**

3.1 Monitoring of pesticide restudies in wastewater

The presence of pesticide residues in the waste drainage water samples, collected from a different location at Al-Jabal Al-Asfar, 1km distance between them in Qalyubia Governorate, Egypt, were determined by GC-MS/MS. From the qualitative and quantitative analyses of pesticide residues, eight pesticide residues were detected as shown in table (1). However, the only frequent residues identified, were N-phosphono methyl glycine (Glyphosate) and Mycobutanil as active compounds, respectively in the year seasons spring, summer, Autumn, and Winter. Pesticides are widely used in agriculture in Egypt, and they are widely recognized as a major source of widespread contamination, mainly through leaching into subsurface aquifers or discharge into surface waters. [25]. In a similar study investigated by Hathout *et al*. [26], 80% of soil samples of Damietta governorate were highly contaminated with pesticides of the different groups belonging to the following chemical groups; triazole (21.43%), organophosphate (10.71%), neonicotinoid (10.71%), pyrethroid (10.71%). Chlorpyrifos-methyl residues were also detected during the summer and autumn seasons during the scrutinizing the different types of water pollutants in El-Mahmoudia water canal, Egypt in 2019 by Radwan *et al*. [27]. In another investigation as a case study in Brazil by da Silva *et al*. [28], Glyphosate was present in all soil samples, with high concentrations reached to 66.38 mg/kg soil.

Table (1): List of the detected pesticides in Al-Jabal Al-Asfar drain water samples that were collected in a year season using the GC-MS/MS

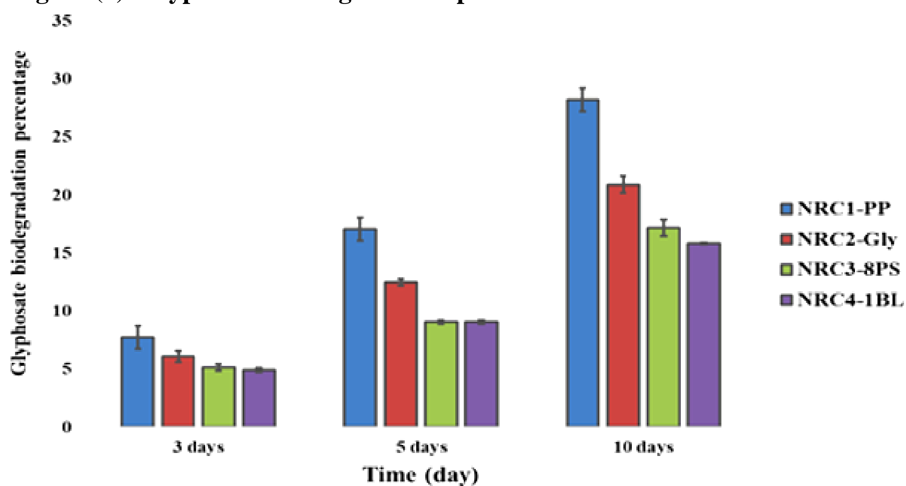
Mean concentration of detected pesticide residues (ng/L)	Time of samples			
	Spring	Summer	Autumn	Winter
Dinotefuran	<LOQ	<LOQ	<LOQ	<LOQ
Dodine	<LOQ	<LOQ		0.010
Halosulfuron	<LOQ	<LOQ	<LOQ	<LOQ
Imidacloprid	<LOQ	0.089	<LOQ	0.022
Mycobutanil	0.010	0.060	<LOQ	<LOQ
Propamocarb	<LOQ	<LOQ	<LOQ	<LOQ
N(phosphonomethyl)glycine (Glyphosate)	0.032	0.068	<LOQ	<LOQ
Clethodiom	<LOQ	<LOQ	<LOQ	<LOQ
Ethylene Thiourea	0.010		<LOQ	
Metalaxyl	<LOQ	<LOQ	<LOQ	<LOQ

LOQ (ngL-1)

3.2 Evaluation the Glyphosate Biodegradation by the bacterial isolates

Only four bacterial isolates took codes NRC1-pp, NRC2-Gly, NRC3-8PS, and NRC4-1BL, succeed in Glyphosate degradation with different potentials as follows: 28.2 % for NRC1-PP, 20.9% for NRC2-Gly, 17.2% for NRC3-8PS, 15.8% for NRC4-1BL after incubation for ten days Fig (2). These isolates were subjected to molecular identification; NRC1-PP was the most potent isolate which degrade (28.2%) of Glyphosate comparable with other bacterial isolates; however, it was subjected to random mutagenesis to improve Glyphosate degradation activity.

Figure (2): Glyphosate biodegradation profile of selected bacterial isolates



3.3 Isolation and molecular identification of glyphosate degradation bacteria

Fifteen bacterial isolates were successfully isolated from drainage wastewater and soil moisture around them. Only four isolates were able to grow rapidly and display the highest glyphosate utilization capability in minimal salt agar enriched with glyphosate pesticide as the sole carbon source. These isolates were subjected to molecular identification by 16S rDNA gene sequencing. Primers of 8F and 1492R succeed in amplification of 1500 bp DNA fragment that represents the 16s rDNA fragment Fig (3). The nucleotide sequences for each isolate 16S rDNA fragment were compared with all available data in the Genbank data base. The aligned results showed that NRC1-PP bacterial isolate exhibited 98.97% homology with *Bacillus cereus* H4, NRC2-Gly isolate had 99.6% homology with *Pseudomonas alcaligenes* strain TIL_TAL_105, NRC3-8PS bacterial isolate had 99.6% homology with *Pseudomonas stutzeri* strain AZ101, besides *NRC4-1BL* isolate had 99.5% homology with *Bacillus licheniformis* strain IND706. The four nucleotide sequences were deposited in the Genbank database under accession numbers MZ031399, MZ031400, MZ031401 and MZ031402 respectively with scientific names of these bacterial isolates as shown in Table (2). The phylogenetic tree of isolates was constructed by MEGAX (Fig.4). From the phylogenetic trees, *Bacillus cereus* strain NRC1_PP was grouped closely with the *Bacillus cereus* strain NR_115526.1, with 98.97% sequence similarity. Also, both NRC2-Gly, and NRC3-8PS were belonged to *Pseudomonas* group as *Pseudomonas alcaligenes* strain NRC2-Gly, *Pseudomonas stutzeri* strain NRC3-8PS with similarity 98.10 and 92.50%

respectively and *Bacillus licheniformis* NRC4-1BL strain with 96% with *Bacillus licheniformis* _NR_118996.Several studies have reported certain bacteria and fungi that exhibited the ability to degrade Glyphosate such as *Pseudomonas* sp. K3, *Alcaligenes sp.* K1, *Azomonas sp.* K5, *Enterobacter cloacae* K7 and *Comamonas sp.* K4, that grew on 10 mM glyphosate concentrations, Kryuchkova *et al.* [29]. Ezaka *et al*. [30] purified some plant growth-promoting bacteria like *Bacillus cereus* and *Pseudomonas aeruginosa*. Acosta-Cortés et al. [31] reported the ability of *B. cereus* 6 P to create polyphosphate as Glyphosate biodegradation through developing a new biotechnological approach. Also, Firdous *et al.* [32] purified a novel bacterial strain *Comamonas odontotermitis* P2, which is proficient in degrading Glyphosate. And Elarabi *et al* . [11] reported that ten isolates were able to survive at a medium containing Glyphosate with different concentrations from (50 to 250 mg/mL) *Bacillus aryabhattai* FACU bacterial isolate showed the highest CFU in all concentrations of Glyphosate. Also, thirteen bacterial isolates belong to the genera *Achromobacter*, Agrobacterium, and *Ochrobactrum* which can consume Glyphosate as the only phosphorous source from the Argentine vastly productive glyphosate-resistant soybean crop area Masotti *et al*.[33]. Furthermore, Mousa *et al*.[34] who also used *Bacillus megaterium* to degrade glyphosate (5-25 ppm) the highest degradation ratio of 70% per 25 ppm for two months.

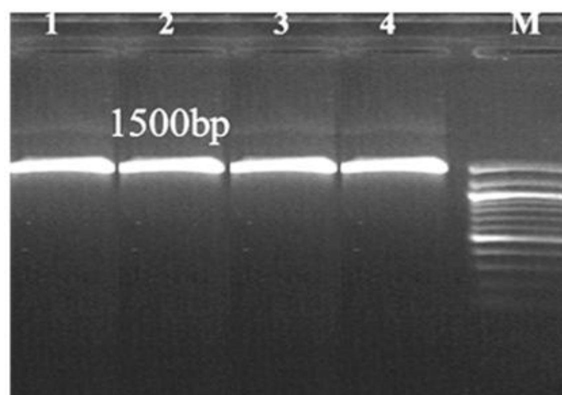
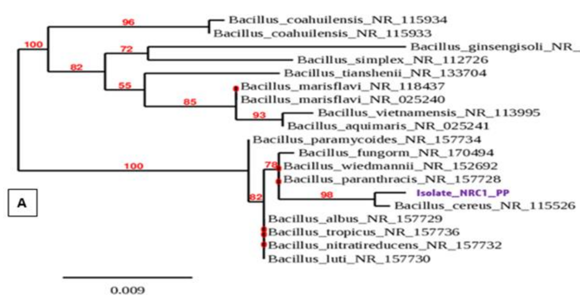
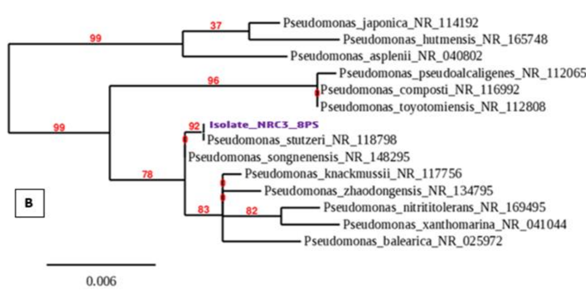


Figure (3): PCR amplification of 16srDNA for bacterial isolates 1500bp lan (1-4) M: 100 bp DNA ladder (thermo scientific)

Table (2) list of selected identified microorganisms with identity percentage and accession numbers

code	Strain	Isolation source	Identity percent	Accession number
NRC1_PP	*Bacillus cereus*	Soil	98.97%	MZ031399.1
NRC2-Gly	*Pseudomonas alcaligenes*	Soil	98.10%	MZ031400.1
NRC3-8PS	*Pseudomonas stutzeri*	Soil	92.50%	MZ031401.1
NRC4-BL	*Bacillus licheniformis*	Soil	96.50%	MZ031402.1





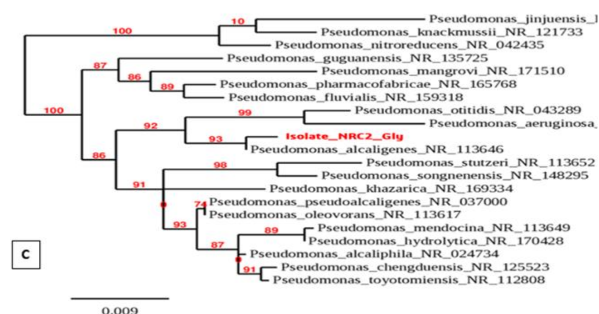


Figure (4): Phylogenetic tree based on 16S rDNA sequences, showing the relationship between isolate [A] Isolate NRC_PP *Bacillus cereus,* [B] Isolate NRC38PS *Pseudomonas stutzeri,* [C] Isolate NRC2_Gly *Pseudomonas alcaligenes*, [D] Isolate NRC_BL *Bacillus licheniformis* and other species belong to the genus *bacillus*.

3.4 Mutation Induction in *Bacillus cereus* strain NRC1-PP

After mutation induction for *Bacillus cereus* strain NRC1-PP using ultraviolet irradiation, viable colonies were counted from appropriate dilution, survival percentage was calculated and the results were depicted in Fig 5 (A). It was noticed that the survival percentage decreased as the exposure time increased. The viability of the cells decreased to 70% when exposed for three minutes until reached 2% at an exposure time of ten minutes, while the exposure time of fifteen minutes was lethal. UV-irradiation with short wavelengths (254 nm) affects the DNA of organisms mainly by the formation of thymine-thymine dimmers and the lethality may be due to the incapability of mutants to repair this damage. In the case of mutation induction by Ethylmethane sulfonate (EMS), also the viability of the cells decreased however by more modest than recorded by UV irradiation. The viability of the cells decreased suddenly to 52.38% at a concentration of 100µL/mL and thereafter diminished up to 1.2% at a concentration of 300µl/ml, while a concentration of 400 µL/mL and 500µL/mL were lethal, Fig 5(B). EMS as an alkylating agent acts primarily on purine bases, where it converts especially guanine to adenine and modifies the DNA [35]. Many studies reported the lethal effects of Ethylmethane sulfonate and UV irradiation as mutagen when used in different concentrations and exposure times [36, 37]. All the bacterial colonies were picked and transferred to minimal salt media containing Glyphosate (300 µg/mL) as the only source for carbon and nitrogen and incubated for three days 30°C. the biodegradation fitness for all mutants was assessed and only one mutant from each treatment exhibited the highest biodegradation capability was selected for evaluating the behavior of biodegradation through time shift.

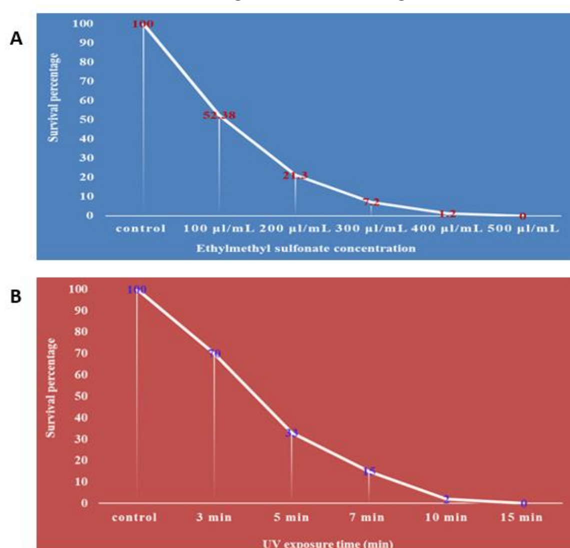


Figure (5): Survival percentage of *Bacillus cereus* strain NRC1-PP after mutation induction by UV irradiation (A) and Ethylmethane sulfonate (B)

3.5 Evaluation the behavior of *Bacillus cereus* strain NRC1-PP and its higher mutants in OPs degradation through time shift

Even though genetic engineering has substantially improved bacterial enzymes, random mutagenesis remains a cost-effective way of generating dependable short-term strains and is widely considered the method of choice. In this study, physical and chemical mutational genetics was assigned to improve the ability of the bacterial strain of *Bacillus cereus NRC1-PP* in Glyphosate biodegradation. All the bacterial colonies that could develop after being exposed to mutation induction, whether it was ultraviolet irradiation or Ethylmethane sulphate, were chosen. Only the mutants that could grow on minimal salt media containing Glyphosate (300 µg/mL) were assessed for biodegradation ability through time shift. About fifty-three and forty-one bacterial colonies were developed after exposure to UV irradiation and EMS, respectively. For thirty days, these selected mutants were allowed to grow on MSM containing 300 µg/mL Glyphosate as a carbon source, and the biodegradation activity was determined. The most notable biodegradation abilities were recorded after three, five, and ten days, indicating that the biodegradation ability did not record detectable values prior to three days and did not increase in biodegradation after ten days. From each mutation induction treatment, the most potent mutants in Glyphosate biodegradation were selected. UV-3, UV-5, UV-7, and UV-10 are symbols that indicate the mutants developed after UV mutation induction for three, five, seven, and ten minutes, respectively. Whereas EMS-100, EMS-200, and EMS-300 are symbols that indicate the mutants developed after EMS mutation induction of concentrations of 100, 200, and 300 µl/ml. From fig (6,7). All these mutants recorded higher Glyphosate biodegradation than the wild strain of *Bacillus cereus* strain NRC1-PP. It was noticeable that UV-10 exhibited the best biodegradation profiles; it recorded the highest percentage of Glyphosate degradation as compared to the other mutants after incubation for three, five, and ten days with values of 20.9%, 51.3%, and 71.9%, as compared with 7.5%, 17.9%, 28.7% in case of wild, respectively. The increase in Glyphosate biodegradation in the highest mutants could be due to several factors; including overexpression of the gene encoding the enzyme responsible for OPs biodegradation, increased secretion system efficiency, or changes in one or more amino acids of the enzyme resulting in increased binding efficiency between produced enzyme and its substrate, which in turn could lead to

increased biodegradation activity. In a previous study by Abo sereih *et al.* [38], they mutated the strain of *Pseudomonas aeruginosa* GH2NO8 by Ethylmethane sulfonate for the first and second steps to evolve a potential mutant, PAMS9, able to degrade 86.21% of diazinon as compared to the wild type with 38.19 % after 5 days. Indeed, mutations alter the structure of proteins, which almost always leads to a loss of function; therefore, structural components changed by mutation are occasionally, improved [39]. Another study by Ibrahim *et al*. [40] employed UV irradiation was employed to improve Mtagse production from *Bacillus cereus*. This is the first study to use chemical and physical mutagenic genetics to improve the biodegradation profile of glyphosate in *Bacillus cereus*. It resulted in efficient OPs-degrading bacterial mutants, which helped to boost OPS detoxification. Xie *et al*. [41] employed (error-prone PCR), the most widely used random mutagenesis approach, to evolve MPH (methyl parathion hydrolase) and improve its effectiveness in chlorpyrifos hydrolysis for OP chemical decontamination. Kalahroudi *et al* [42] also announced that strain improvement via UV treatment is a beneficial and practical method for increasing protease production in significant manner in *L. enzymogenes* in comparison to wild type strains.

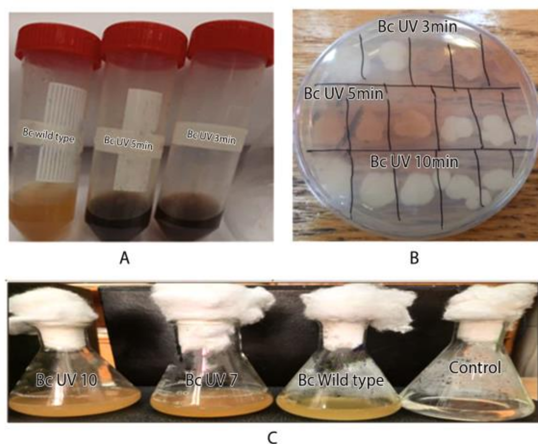


Figure (6): Bacterial growth of the wild *Bacillus cereus* strain NRC12 and its mutants A: The growth of wild its brown mutants on LB broth media. B: The growth of wild and its brown mutants on LB agar plats. C: Bacterial growth of the Bacillus cereus strain NRC12 and its mutants on liquid MSM containing Glyphosate (300 µg/mL) after 10 days.

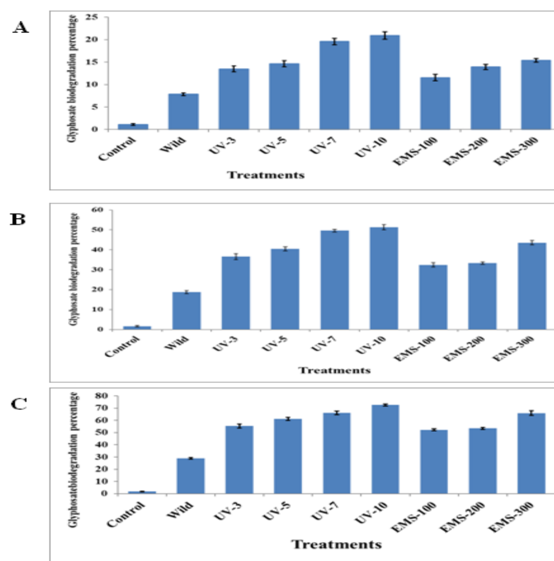


Figure (7): Glyphosate biodegradation by *Bacillus cereus* strain NRC1-PP and its higher mutants after three days (A), after five days (B), after then days(C).

3.5 Glyphosate metabolites

The Mass Hunter [M–H]+ ions of GLYP (m/z 340), reflected the MS spectra of GLYP), with no significant fragmentation evident (Figure 8 A and B). the lack of fragmentation proved the high-quality MS spectra analysis according to Evans et al [43, 44], he inferred that relative and absolute ions abundance is affected by fragmentation. There were different types of metabolites due GLY degradation in control and treated samples. Metabolites reported in control sample were C2N2O , C3H4O3P, Tert-butyl, 2-Amino 3-phosphoo propionic, C4H7NO6P, AMPA, AMPA + methyl, glyphosate, [((CH3)3SiO)2PO(CH3)2Si]+, (CH3)3SiOCOCH2N ((CH3)3Si)CH2]+, ((CH3)3SiO)2PO(CH3)3Si] and CH3)3SiOSi(CH3)2, where as in case of treated sample, CH5O3 P, [PO(OCH3)2, Methyl phosphino propionic, Sarcosine, C8H6NO3P , C5H3 P, Amino methyl phosphonic acid and AMPA + methyl were reported, tables (3,4). The mass to charge ratio (m/z) of value 370 referred to loss of a water molecule from either the carboxyl or the phosphonic acid group. The primary natural metabolite of GLYP, AMPA was appeared at m/z 168 and that was also reported by Goodwin et al. [45]. The formation of phosphorus-containing anions was arisen by the major analytes at m/z 340. Moreover, there was a common feature that reflected the loss of CO_2 in the spectra of the carboxyl-containing molecules as shown in (Fig.9, 10).

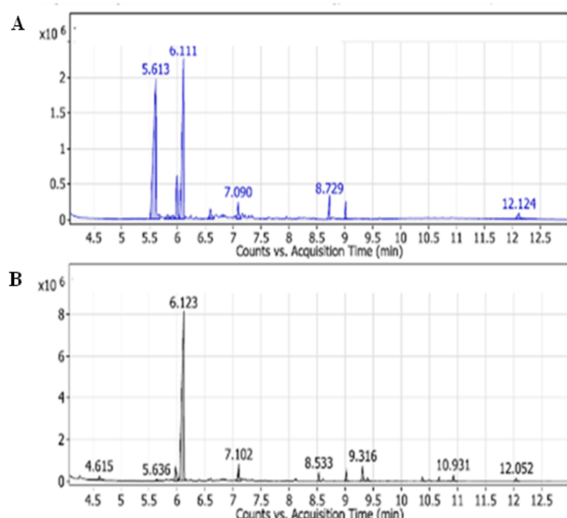


Figure (8): full scan of Glyphosate metabolites by GC-MS, (A) in control sample (B) in treated sample with the best mutant(UV 10) of *Bacillus cereus*

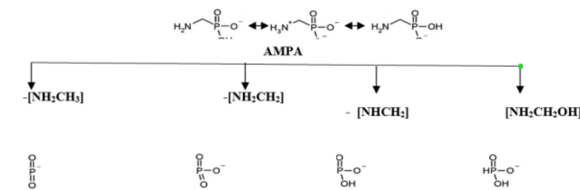


Figure (9): Fragmentation map for GLYP Structures suggested based on rationalization of MS fragmentation.

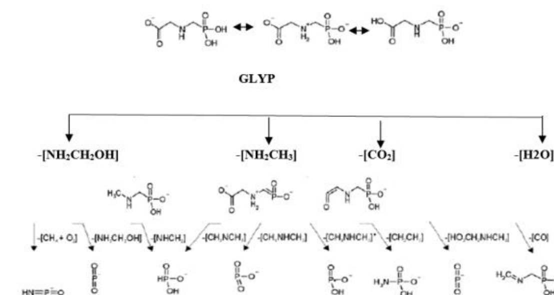


Figure (10): Fragmentation map for AMPA Structures suggested based on rationalization of MS fragmentation.

Table 3: Glyphosate with mutated *Bacillus cereus*UV10 in free M.S.M

No	Retention time (min)	Glyphosate	m/z	Fragments
1	4.6	C2N2O + 3(TMS)	68+221	289.1,221,207,175,133,91,73,59,15
2	5.6	C3H4O3P+ TMS	133(M+1)	207,155.1,127.1,99.,85.1,57.1, 32
3	5.9	Tert-butyl+ 3 TMS	57(M+1)	278.2,263.2,247.1,207.1,115.1,91.1,73.1,57.141.1
4	6.0	2-Amino 3-phosphoo propionic	169(M+2)	171.0,129,103.0,75,43.0
5	7.07	C4H7NO6P +TMS	196 (M+1)	197.1, 180.1, 139.1, 115.0, 91, 77, 51, 32
6	8.5	AMPA	312(M+1)	313.1,299,269.3,227,201.1,171,132, 117,95.173,43.1
7	9.0	AMPA + methyl	326(M+1)	327, 311,255,227,185,157, 129,103,75,43
8	9.4	Glyphosate	340(M+1)	341.3,314.2,226.2,201.1,165.1, 145.1,132,117,97.1,75.1,41.1,32
9	10.3	[(CH3)3SiO)2PO(CH3)2Si]$^{+}$	283(M+2)	285,236.2,221.1,205.1,1611, 133.1,91.1,73.1,57.1,32.0
10	10.6	[(CH3)3SiOCOCH2N (CH3)3Si)CH2]$^{+}$	232(M+1)	233.1,205.1,147,91,73,57,32
11	10.9	[(CH3)3SiO)2PO(CH3)3Si]	298(M+1)	371(-TMS),313,257,239, 203,147,103,73,57,43
12	12	CH3)3SiOSi(CH3)2	147(M)	147,129,73,32

Table 4: Glyphosate without bacteria in free M.S.M

No	Retenti on time (min)	Glyphosate	m/z	Fragments
1	5.5	CH5O3 P	96 (M+1)	97.1,83.1,69.1,55.1,41.1
2	5.9	[PO(OCH3)2	109 (M+2)	111.1,85.1,69.1,55.1,43.1,32.1
3	6.0	Methyl phosphino propionic	169 (M+2)	71.1,129.1,103.1,83.1,75,55.141.1
4	6.6	Sarcosine	89(M+2)	91.1,77.1, 51.1,32
5	7.0	C8H6NO3P	196 (M+1)	197.1,180.1,165.1,153.1,111.1,77.0,57.1,43.1,32.1
6	8.7	Amino methyl phosphonic acid	111 (M)	111,97,83,69,55,29,19
7	9.0	AMPA + methyl	326(M+1)	327.4,311.1,252.2,227.2,185.1,157.1,129,97.1,75.1,43.1
8	12	C5H3 P	94 (M+1)	95.1,55.1

3.6 SDS-PAGE analysis of *Bacillus cereus NRC1-PP* and their mutant:

SDS-PAGE of whole cell proteins has been described by many researchers as a powerful technique for strain discrimination. SDS-PAGE profile analysis was employed to determine the distances/similarities between parental and putative mutant strains. The results of SDS-PAGE of whole-cell proteins of *Bacillus cereus* and its mutants were

illustrated in figure (11). The total number of bands was 80 bands. The molecular weight of these bands ranged from 7.5 to 280 KD. It showed that ten polymorphic protein bands at the molecular size of 280, 250, 130, 100, 70, 55.6, 35, 25, 18.55, and 7.5 KD. It showed one positive unique protein band for (M1) at a molecular size of 73 KD. Three monomorphic protein bands were determined at fragment sizes of 20, 15, and 10 KD for all mutant and wild type. Also, there are two negative unique protein bands for mutant (M4 and M6) at molecular weight 35 and 70 KD. Data presented in table (5) explained that polymorphism percentage (P%) and polymorphism information content (PIC) for pattern generated by SDS-PAGE and the result indicated polymorphism percentage (%) was 71.42 % for all polymorphic bands and total bands generated from SDS-PAGE pattern. The number of total fragments produced from all isolates in this pattern was 80 fragments, each isolate WT, M1, M2, M3, M4, M5, M6, M7 was produced 11, 10, 12, 11, 5, 12, 6 and 13 fragments, respectively. Also, the polymorphism information content (PIC) was calculated as 0.435 generated from the SDS-PAGE pattern. Therefore, our result was similar with Salam and Hotzel, [46] were reported that the genetic similarity and protein profile of the isolated field German strains was compared to *Mycoplasma bovis* reference strain using SDS-PAGE. In addition, Hafeez and Sultana [47] investigated the whole cell protein profile of 20 isolates that were taken from the NIH Islamabad using SDS-PAGE to determine the variability in the gene pool of *E. coli*. As shown in Figure (12) Principal component analysis (PCA) of isolated bacterial strains using SDS-PAGE included three clouds A, B1 and B2. Principal component analysis (PCA) is a powerful tool and the most basic multivariate data reduction statistical technique. The results of PCA revealed that two out of six principal components were significant (Eigen value >1) and contributed 77.24% of the total variation. PC 1 accounted for 58.97 % and PC 2 accounted for 18.26% of the total variation. PCA found the same grouping pattern as found in the cluster analysis, indicating that significant variation exists in this study. The separation of PC 1 and PC 2 showed that seven bacterial mutants were dispersed in all quarters, indicating a high level of genotypic variation. The numerical analysis clearly revealed two distinct clusters as shown in the dendrogram; it was classified into two main clusters (A and B). cluster A includes two mutant (M4 and M6), and cluster B include two main sub clusters (B1 and B2), Sub cluster B1 contain only one mutant M1 was separated from sub cluster B2 that contains (M2, WT, M3, M5, and M7) these results were similar to Jamalzadeh *et al*. [48] who reported that genetic diversity and plant growth-promoting activity of the dominant bacteria from plants in Western Iran.

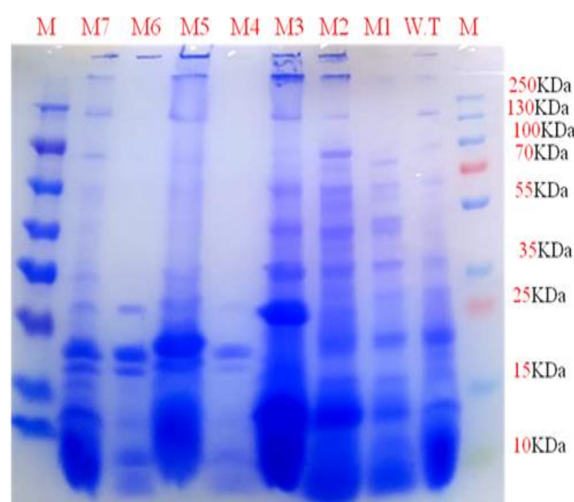


Figure (11): Patterns of SDS-PAGE electrophoretic protein of selected mutant and wild type, Lane1: protein ladder (PageRuler™ Plus Pertained), Lane2: wild type (WT), and lanes (3 : 9) represent mutant from M1 – M7.

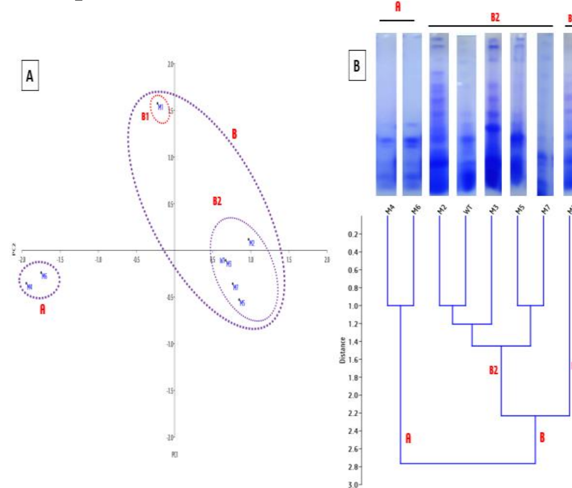


Figure (12): (A) Principal component analysis (PCA) of Bacillus *cereus* and selected mutants: (B) Dendrogram of SDS-PAGE based on unweighted pair group method with arithmetic averages algorithm (UPGMA) for *Bacillus cereus* strain and its mutants

Table 5: Polymorphism generated by SDS-PAGE of the selected mutant with wild type.

MW	WT	M1	M2	M3	M4	M5	M6	M7	Frequency	Polymorphism	P. B	M.B	Unique	T. B	P%	PIC
280.0	1	0	1	1	0	1	0	1	**62.5**	**Polymorphic**	**10**	**3**	**1**	**14**	**71.42**	**0.435**
250.0	1	0	1	1	0	1	0	1	**62.5**	**Polymorphic**						
130.0	1	0	1	1	0	1	0	1	**62.5**	**Polymorphic**						
100.0	1	1	1	0	0	1	0	1	**62.5**	**Polymorphic**						
73.00	0	1	0	0	0	0	0	0	**12.5**	**Unique**						
70.00	1	1	1	1	0	1	0	1	**75.0**	**Polymorphic**						
55.60	0	1	1	1	0	1	0	1	**62.5**	**Polymorphic**						
35.04	1	1	1	1	0	1	0	1	**75.0**	**Polymorphic**						
25.00	1	1	1	1	0	1	1	1	**87.5**	**Polymorphic**						
20.00	1	1	1	1	1	1	1	1	**100**	**Monomorphic**						
18.55	0	0	0	0	1	1	1	1	**50.0**	**Polymorphic**						
15.00	1	1	1	1	1	1	1	1	**100**	**Monomorphic**						
10.00	1	1	1	1	1	1	1	1	**100**	**Monomorphic**						
7.50	1	1	1	1	1	0	1	1	**87.5**	**Polymorphic**						
Total	**11**	**10**	**12**	**11**	**5**	**12**	**6**	**13**	80 bands							

PB number of polymorphic bands, MB number of monomorphic bands, TB number of total bands, %P percent polymorphism (PIC) polymorphism information content

4. Conclusion

Microorganism-based technology is regarded extremely efficient, low-cost, and eco-friendly for remediating contaminated environment, highlighting the importance of identifying new bacterial strains capable of degrading Glyphosate to perform its bioremediation. UV mutagenesis has been regarded as the most promising tool to generate more potent mutants than the wild type. *B. cereus* NRC1-PP and its mutants are considered good candidates for Glyphosate biodegradation.

5. Conflicts of interest

There are no conflicts to declare.

6. Acknowledgments

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