

Bioremediation of Glyphosate by Bacteria Isolated from Glyphosate Contaminated Soil

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Glyphosate is an organophosphorus insecticide widely used as a herbicide. The intensive use of herbicides in agricultural soils is a matter of environmental concern. Glyphosate is toxic to environment, exploration of various glyphosate degrading bacteria to clean-up the pesticides is of immense importance. Four bacterial isolates were successfully isolated from glyphosate contaminated soil and were named GP-1, GP-2, GP-3 and GP-4. The isolates were capable of utilizing glyphosate as the sole source of carbon and were able to grow at 1000 mg/l concentrations of glyphosate. Effect of environmental conditions like temperature, pH and NaCl concentration was determined for all bacterial isolates. Most of bacterial isolates showed susceptibility for different antibiotics in antibiotic sensitivity test. Growth kinetics experiments showed that the bacterial isolates were able to grow in medium containing the glyphosate as the carbon source. Out of four bacterial isolates GP-4 was identified based on 16S rRNA sequence analysis.

Key words: Glyphosate, Organophosphorus, Growth kinetics, 16SrRNA

Agriculture is the lynchpin of the Indian economy. Ensuring food security for more than 1 billion Indian population with diminishing cultivable land resource necessitates use of high yielding variety of seeds, balanced use of fertilizers and judicious use of quality pesticides. Pesticides are the chemical substances that kill pests like fungi, insects, worms, and nematodes etc. which cause damage to field crops. Pesticides are used as a leading mean to control variety of weed species, harmful insects and approximately 1500 plant diseases. The excessive use of pesticides leads to an accumulation of a huge amount of pesticide residues in the food chain and drinking water environment that further leads to a substantial health hazard for the current and future generations due to uptake and accumulation of these toxic compounds.

The molecule *N*-(phosphonomethyl) glycine was first synthesized in 1950 by a researcher of the small Swiss pharmaceutical firm Cilag, Henri Martin. Yet, showing no pharmaceutical perspective the compound has not been investigated any further. A decade later through the acquisition of the company it was transferred to the distributor of laboratory research chemicals, Aldrich Chemical Co. along with research samples of Cilag. This is how it came to the attention of Monsanto Company in the course of its research to develop phosphonic acid type water softening agents through testing over 100 chemical substances related to aminomethyl phosphonic acid (AMPA). Monsanto later extended the study of these compounds to herbicide activity testing and observed their potential against perennial weeds. *N*-(phosphonomethyl) glycine was first re-synthesized and tested by Monsanto in 1970. Its herbicidal effect was described by Baird and co-workers in 1971, the subsequent patent (US 3799758), followed by numerous others was claimed

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and obtained by Monsanto and was introduced as a herbicide.

Glyphosate is a non-selective, systemic herbicide that can control most annual and perennial plants. Glyphosate is usually formulated as an isopropylamine salt. While it can be described as an organophosphorus compound glyphosate is not an organophosphate ester but a phosphanoglycine and it does not inhibit cholinesterase activity. It controls weeds by inhibiting the synthesis of aromatic amino acids necessary for protein formation in susceptible plants. Glyphosate is strongly adsorbed to soil particles which prevents it from excessive leaching or from being taken-up from the soil by non-target plants. Glyphosate is degraded primarily by microbial metabolism but strong adsorption to soil can inhibit microbial metabolism and slow degradation. Photo and chemical degradation are not significant in the dissipation of glyphosate from soils. Glyphosate is moderately persistent with a half-life of 30–170 days. In water glyphosate is rapidly dissipated through adsorption to suspended and bottom sediments and has a half-life of 12 days to ten weeks. Pesticides and their degradation products generally get accumulated in the top soil and influence not only the population of various groups of soil microbes but also their biochemical activities like nitrification, ammonification, decomposition of organic matter and nitrogen fixation (Agnihotri *et al.*, 1981). Microorganisms play an important role in degrading synthetic chemicals in soil (Alexander, 1981). They have the capacity to utilize virtually all naturally and synthetically occurring compounds as their sole carbon and energy source.

Bioremediation constitutes an attractive alternative to physico-chemical methods of remediation, as it is less expensive and can selectively achieve complete destruction of organic pollutants. In bioremediation microbes that can degrade the pesticides *in situ* are used. For a successful bioremediation technique an efficient bacterial strain that can degrade largest pollutant to minimum level is required. Microbial degradation is considered to be the most important of the transformation processes controlling its persistence in soil (Araujo *et al.*, 2003).

Microbial degradation of glyphosate produces the major metabolite aminomethyl

phosphonic acid and ultimately leads to the production of CO₂, phosphate and water (Forlani *et al.*, 1999). Several species of bacteria have been isolated from previously treated and untreated environments which can degrade glyphosate either co-metabolically or as a source of phosphorus. Several species of *Pseudomonas* have been isolated which can degrade glyphosate (Moore *et al.*, 1983). Several bacterial strains were isolated that were able to convert glyphosate to sarcosine and inorganic phosphate under phosphorus limiting conditions but such ability was lost when other P sources were made available (Dekker and Duke, 1995). A strain of *Arthrobacter atrocyaneus* was indeed reported to metabolize glyphosate to AMPA (Pipke and Amrhein, 1988). Similarly a *Flavobacterium sp.* (Balthazor & Hallas, 1986), an *Alcaligenes sp.* (Tolbot *et al.*, 1984), *Bacillus megaterium* strain 2BLW (Quinn *et al.*, 1989), several species of *Rhizobium* (Liu *et al.*, 1991), three species of *Agrobacterium* (Liu *et al.*, 1991) and an *Arthrobacter sp.* (Pipke *et al.*, 1987) have also been reported to degrade this herbicide.

MATERIAL AND METHODS

Sample collection

Soil samples were collected from the sludge produced by glyphosate producing industrial plants of Ahmedabad, Ankleshwar, Vadodara, Kalol and different parts of Gujarat. Soil samples were also collected from field where glyphosate has been applied for more than 3 years for the control of various insect pests. The collected samples were air dried, ground, passed through 2 mm sieve and stored in the sealed plastic bags at room temperature. These stored samples were used for further experimentation.

Pesticide used

Glyphosate technical (97% purity) was obtained from Crop Life Science Limited, Ahmedabad and glyphosate standard (99% purity) was purchased from Sigma-Aldrich, USA.

Enrichment of soil samples

Soil samples were air dried to 20% (w/w) moisture content and passed through a sieve with 2mm mesh. Soil samples were enriched in 250 ml erlenmeyer flasks containing 50 ml MSM supplemented with glyphosate (250 mg/l) and 10 g soil. For enrichment of glyphosate degrading

bacteria MSM was supplemented with glyphosate (250 mg/l) as a sole source of carbon, energy. The flasks were incubated on a rotary shaker at 150 rpm for seven days at 30°C. After a week, 1 ml from the above medium was inoculated to the same fresh medium. This was repeated 5-6 times for selective enrichment of glyphosate degrading bacteria.

Isolation and screening of glyphosate degrading bacteria

For isolation of glyphosate degrading bacteria the enriched soil samples were serially diluted and spread plated on to MSM containing glyphosate (250 mg/l). After isolation, the representative microorganisms growing on the plates were purified following the four-way streaking method. The glyphosate degrading bacterial isolates were tested for their ability to grow on higher concentration by inoculating to the MSM containing chlorpyrifos (500, 750 and 1000 mg/l) with and without agar. Finally, the strains with the tolerance to the highest chlorpyrifos concentration were selected and different biochemical tests were performed for further identification.

Effect of environmental conditions on bacterial growth

There are many parameters which affect growth of bacterial isolates viz. temperature, pH, aeration, salt concentration, nutrient availability, radiation, presence of heavy metals, carbon source, etc. Out of these, some important parameters viz. pH, temperature, salt concentration affecting bacterial growth were considered.

Effect of temperature on bacterial growth

Effect of temperature on bacterial growth was tested on both nutrient agar medium and nutrient broth. All bacterial isolates were inoculated and incubated at different range of temperature 15°C, 25°C, 35°C, 45°C and 55°C for 48 hr. After incubation presence or absence of growth on nutrient agar medium was recorded. The absorbance of bacterial isolates from nutrient broth was measured at 600 nm.

Effect of pH on bacterial growth

Effect of pH on bacterial growth was tested on both nutrient agar medium and nutrient broth. Nutrient agar medium and nutrient broth were adjusted with different ranges of pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. All bacterial isolates were

inoculated and incubated at 37°C for 48 hr. After incubation presence or absence of growth on nutrient agar medium was recorded. The absorbance of bacterial isolates from nutrient broth was measured at 600 nm.

Effect of NaCl on bacterial growth

Effect of NaCl on bacterial growth was tested on both nutrient agar medium and nutrient broth. Nutrient agar medium and nutrient broth were supplemented with different ranges of NaCl concentration 1.0, 2.0, 3.0 and 4.0 %. All bacterial isolates were inoculated and incubated at 37°C for 48 hr. After incubation presence or absence of growth on nutrient agar medium was recorded. The absorbance of bacterial isolates from nutrient broth was measured at 600 nm.

Antibiotics sensitivity test

Sensitivity of bacterial isolates to antibiotics was tested on N-agar medium using the octadiscs of antibiotics (Himedia manufactured). All glyphosate degrading isolates were inoculated in nutrient broth and incubated at 37°C for 24 hr. After incubation 100 µl of bacterial culture was spreaded on nutrient agar plate and octadiscs of antibiotics were kept on inoculated plates. The plates were incubated at 37°C for 48 hr. The zone of inhibition was observed for each disc. The following antibiotics were used for sensitivity test: Tetracycline (TE), Chloramphenicol (C), Ampicillin (Amp), Gentamicin (Gen), Cefazolin (Cz), Cefuroxime (Cxm), Amikacin (Ak), Co-Trimoxazole (Cot).

Growth Kinetics

All bacterial isolates were inoculated in MSM supplemented with two different concentrations of glyphosate (500 and 1000 mg/l). The MSM inoculated with bacterial isolates were incubated at 37°C in shaking condition (120 rpm). The MSM containing glyphosate (500 and 1000 mg/l) and *Escherichia coli* which is not able to degrade glyphosate was used as the negative control. The growth kinetics was followed by monitoring the optical density of the medium for 9 days using a UV/VIS spectrophotometer at 600 nm wavelength.

Identification of bacteria

Out of all bacterial isolates GP-4 was characterized based on 16S rRNA gene analysis. The genomic DNA was extracted using phenol-chloroform method. The 16S rRNA gene was

amplified and sequencing was carried out with an automated sequencer (ABI 3130 XL).

16S rRNA sequences were compared to other 16S rRNA sequences available in the National Center for Biotechnology Information (NCBI) public database by basic local alignment search tool (BLAST) searching. Selected sequences from the database with the greatest sequence similarity to isolated bacterial sequence were aligned and compared.

RESULTS AND DISCUSSION

Isolation, screening and biochemical characterization of isolates

During primary screening four strains were isolated that were capable of utilizing glyphosate (250 mg/l) as the sole source of carbon. The isolates designed GP-1, GP-2, GP-3 and GP-4 were grown in different concentrations of glyphosate (500, 750 and 1000 mg/l). Biochemical studies were carried out (Table 1) according to Bergey's Manual of Systematic Bacteriology (Vol I and II).

Effect of environmental conditions on bacterial growth

All four isolates showed ability to grow at wide range of temperature, pH and NaCl concentration. The effect of temperature on

bacterial growth was seen in the temperature range of 15°C to 55°C with the maximum growth seen at 35±2°C. All isolates were unable to grow at 45°C and 55°C whereas GP-1 was unable to grow at 15°C. The effect of pH on bacterial growth was observed in the pH range of 4 to 10 with the maximum growth seen at pH 7. All four isolates were unable to grow at pH 4 whereas GP-3 was unable to grow at pH 10. The optimum NaCl concentration for all four isolates was found to be 1 %. All isolates other than GP-3 were grown up to 4 % NaCl concentration. GP-3 was able to grow upto 3 % NaCl concentration.

Antibiotic sensitivity

For antibiotic resistance/ susceptibility profiling, the disc diffusion method was used. The zone of inhibition was measured in millimeter and the resistance and sensitivity of isolated bacteria towards antibiotics used was determined. All four isolates were found susceptible to Tetracycline, Co-Trimoxazole, Amikacin, Gentamycin, and Chloramphenicol. GP-1 and GP-2 were resistance while GP-3 and GP-4 were susceptible to Cefuroxime. All four isolates were resistance to resistance Cefazolin while all bacteria other than GP-2 were resistance to Ampicillin.

Growth kinetics

Bacterial growth in MSM supplemented with two different concentrations of glyphosate

Table 1. Biochemical test results

Organism	Citrate	Starch	Motility	Oxidase	Catalase	H ₂ S	Indole	Nitrate	Acid	Gas	Gram	Urease
GP-1	-Ve	-Ve	-Ve	+Ve	+Ve	-Ve	+Ve	+Ve	-ve	-ve	-Ve	-Ve
GP-2	+Ve	-Ve	-Ve	+Ve	+Ve	+Ve	-Ve	+Ve	-ve	-ve	-Ve	-Ve
GP-3	-Ve	-Ve	-Ve	+Ve	-Ve	-Ve	-Ve	+Ve	-ve	-ve	-Ve	-Ve
GP-4	-Ve	-Ve	-Ve	-Ve	+Ve	-Ve	+Ve	-Ve	-ve	-ve	-Ve	-Ve

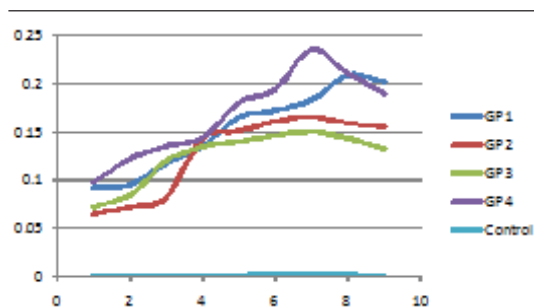


Fig. 1. Growth Kinetics of bacterial isolates in presence of glyphosate (500 mg/l)

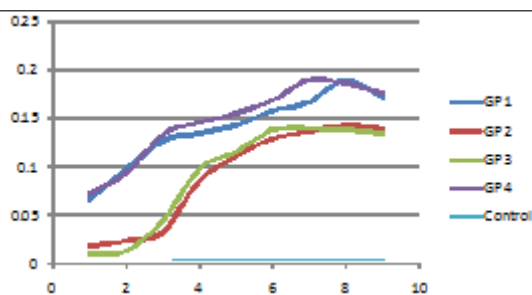


Fig. 2. Growth Kinetics of bacterial isolates in presence of glyphosate (1000 mg/l)

(500 and 1000 mg/l) is shown in figure 1 & 2. As compared to control sample, the growth of all bacterial isolates was significantly stimulated and approximately two to three times faster at the beginning of incubation period. Maximum bacterial growth in 500 mg/l was obtained by day seven in GP-2, GP-3 and GP-4 where as in GP-1 maximum growth was obtained by day eight. In growth kinetics at 1000 mg/l maximum growth was obtained by day eight in GP-1 and GP-2 where as GP-3 and GP-4 showed maximum growth at day seven. The growth curve reached at static after maximum growth and then decreased. In contrast the control sample inoculated with *E. coli* showed no change at 600 nm for 9 days incubation.

Identification of bacteria

For the further identification at strain level GP-4 was identified by 16S rRNA sequencing. 16S rRNA gene sequence of GP-4 was compared with that of referred strains gene sequences in the Genebank. GP-4 shows close homology (98%) with the sequence of *Mycobacterium brisbanense*. The sequence was processed by sequin stand-alone software and deposited it in the Genebank database with accession number KT821090.

CONCLUSION

All bacterial isolates were able to tolerate high concentration of glyphosate. The isolates also showed growth at wide range of temperature, pH and salt concentration. Most of bacterial isolates were sensitive to antibiotics. In growth kinetics study all isolates showed variation in maximum growth time period. 16S rRNA analysis revealed that GP-4 is related to *Mycobacterium brisbanense* which is able to participate in efficient degradation of glyphosate. Thus the capacity of the isolates to survive and grow in the presence of high concentrations of the glyphosate marks them out as good candidates for the bioremediation of glyphosate polluted environments.

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