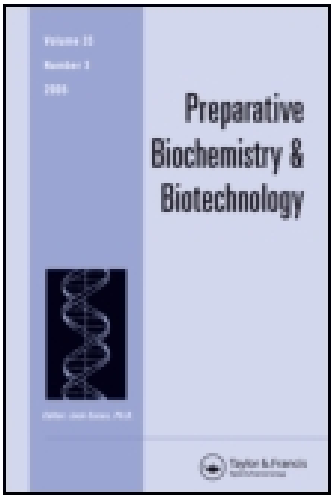

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Purification and Characterization of Carbon–Phosphorus Bond-Cleavage Enzyme From Glyphosate Degrading *Pseudomonas putida* T5

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An inducible, carbon-phosphorus bond-cleavage enzyme was purified from cells of *Pseudomonas putida* T5 grown on *N*-phosphonomethyl glycine. The native enzyme had a molecular mass of approximately 70 kD and upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), yielded a homogeneous protein band with an apparent molecular mass of about 70 kD. Activity of purified enzyme was increased by 627-fold compared to the crude extract and showed pH and temperature optima of approximately 7 and 30°C, respectively. The purified enzyme had an apparent Km and Vmax of 3.7 m*M* and 6.8 m*M*/min, respectively, for its sole substrate *N*-phosphonomethyl glycine. The enzyme was inhibited by phenylmethylsulfonyl fluoride (PMSF), indicating the presence of serine at the active site. The enzyme was not inhibited by SDS, suggesting the absence of disulfide linkage in the enzyme. The enzyme was found to be inhibited by most of the metals studied except Mg^{2+}. Detergents studied also inhibited glyphosate acting as a carbon–phosphorus bond-cleavage enzyme. Thus initial characterization of the purified enzyme suggested that it could be used as a potential candidate for glyphosate bioremediation.

Keywords *bioremediation, carbon-phosphorus bond cleavage enzyme, glyphosate,* N-*phosphonomethyl glycine,* Pseudomonas putida

INTRODUCTION

Organophosphonates, characterized by the presence of a stable, covalent carbon-to-phosphorus (C-P) bond, are of widespread occurrence in the environment. Natural and synthetic organophosphonates are of importance, with the latter being utilized extensively in the chemical industries[1] as pesticides, plasticizers, air fuel ingredients, and chemical warfare agents. Organophosphorus compounds are the most widely used herbicides and insecticides, accounting for an estimated 34% of worldwide insecticide sales.[1]

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Contamination of soil from these pesticides as a result of their bulk handling at the farmyard or following application in the field or accidental release may lead to contamination of surface water and groundwater. Contamination of air, water, and terrestrial ecosystems has been reported to lead to harmful effects on different biota and disruption of biogeochemical cycling by these organophosphorus compounds.[2] Organophosphorus compound poisoning is a worldwide health problem contributing to around 3 million poisonings and 200,000 deaths annually.[3,4] These compounds have been implicated in several nerve and muscular diseases in humans, and their acute adverse effects have been extensively reported by Colborn et al. and Ragnarsdottir.[5,6] Immunotoxicity of organophosphorus compounds in humans and wildlife has been reviewed by Galloway and Handy.[7]

Continuous and excessive use of organophosphorus compounds has led to the contamination of several ecosystems in different parts of the world.[8–10] These compounds possess high mammalian toxicity and it is therefore essential to remove them from the environment. By far the most important use of synthetic organophosphonates, however, is as herbicides, with glyphosate,[11] the world's leading agrochemical, worth in excess of $1 billion per year[1] It is a representative of the phosphonic acid group of compounds, which is characterized by a direct carbon to phosphorus (C-P) bond. The C-P linkage is chemically and thermally very stable and renders the molecule much more resistant to nonbiological degradation in the environment than its analogues with O-P linkage.[12]

Bioremediation can offer an efficient and cheap option for decontamination of polluted ecosystems and destruction of chemical warfare agents.[13] Acclimated bacterial cultures have been tested for their ability to degrade different organophosphorus pollutants.[14]

It was shown that an obligately thermophilic microorganism could cleave C-P bonds and utilize a number of organophosphonates for growth. Microbial degradation has been considered to be the most important of the transformation processes controlling its persistence in soil.[15] It was observed that mineralization of glyphosate is related to both the activity and biomass of soil microorganisms.[16] Several species of *Pseudomonas* capable of degrading glyphosate have been isolated.[17,18]

The biodegradation of the herbicide glyphosate via the sarcosine pathway by microorganisms and also the conversion of glyphosate to amino methyl phosphonic acid (AMPA) have been accepted as mechanisms for detoxification of this herbicide in soil; no microorganism that conclusively exhibited this capability has been isolated from soil.[19] To date, our understanding of this phenomenon is based almost exclusively upon work carried out by Monsanto on microbes within a glyphosate waste treatment plant, which also metabolized the herbicide via this pathway.[20,21]

The present study shows for the first time the conclusive production of sarcosine, which is a nontoxic[22,23] metabolite of glyphosate degradation by a microorganism. Isolations of microorganisms that utilized glyphosate as carbon or nitrogen sources were attempted. Microbes that degrade phosphonates as phosphorus sources have been isolated and characterized. This *Pseudomonas* sp. was observed to grow with glyphosate as a sole phosphorus source with no accumulating intermediates. To our knowledge, a microbe that accumulates sarcosine as an intermediate has not been reported. The purposes of this study were (i) to isolate a medium microorganism capable of degrading glyphosate and (ii) to purify and characterize the first enzyme capable of breaking C-P bonds.

MATERIALS AND METHODS

Materials

N-Phosphonomethyl glycine (glyphosate) was procured from Monsanto Agricultural Co., St. Louis MO. Sarcosine and other pesticides were procured from Serva, Germany. Sepharose was purchased from Pharmacia. All other chemicals used in these studies were obtained from standard chemical companies such as Sigma-Aldrich chemical company, E. Merck Laboratories, and others and were of analytical grade.

Microorganism and Culture Conditions

Pseudomonas putida T5 used in this study was a member of the glyphosate-degrading bacterial consortium.[24,25] This isolate was maintained on nutrient agar and also on minimal agar containing 5 ppm glyphosate.

Screening of the Bacterial Cultures

All the individual strains were grown on nutrient broth for 48 hR at room temperature separately. These were then harvested by centrifugation at 10,000 rpm for 10 min at 4°C; the supernatant was discarded and the remainder was washed in minimal medium (pH 7.5) of the following composition (per liter): KH_2PO_4, 0.675 g; K_2HPO_4, 5.455 g; NH_4NO_3, 0.25 g. The cells were then sonicated in ice-cold phosphate buffer in an ice bath. The harvested cells were resuspended in a known quantity of minimal medium. The cells were preexposed to 10 ppm glyphosate for 72 hr. Preexposure of the cells was done by periodic addition of 10 ppm substrate at every 24 hr. After the preexposure, all the consortial cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C.

Enzyme Production

Pseudomonas putida T5 was grown in batch cultures at 30°C on an orbital shaker at 150 rpm in nutrient broth. After 48 hr of growth, the cells were harvested and resuspended in minimal medium (pH 7.5) of the following composition (per liter): KH_2PO_4, 0.675 g; K_2HPO_4, 5.455 g; NH_4NO_3, 0.25 g. The cells were induced with daily addition of 10 ppm glyphosate up to 72 hr. The flasks were incubated at 30°C on an orbital shaker at 150 rpm.

Enzyme Isolation

The cells were separated from the broth by centrifugation at 4°C, 10,000 rpm, for 15 min. The supernatant was discarded. Cells were washed well in minimal medium. The cells were then sonicated in ice-cold phosphate buffer in an ice bath. The homogenate was centrifuged for

20 min at 10,000× g at 4°C, and the clear supernatant was concentrated using centricon tubes with 10,000 cutoff, dialyzed against phosphate buffer and designated as crude extract.

Assay of Glyphosate C-P Lyase Activity

Quantitative determination of glyphosate C-P lyase was carried out as follows: The assay reaction was carried out in a final volume of 1 mL containing 50 m*M* phosphate buffer (pH 7.0), enzyme, and 60 μ*M* glyphosate. The reaction mixture was incubated at 30°C for 60 min. After the reaction, residual glyphosate was hydrolysed at 36°C with sodium hypochlorite to form glycine. The glycine was then reacted with *o*-phthaldehyde (OPA) in the presence of *β*-mercaptoethanol at 55°C to produce a highly fluorescent isoindole for fluorimetric detection in high-performance liquid chromatography (HPLC).

HPLC was carried out using a cation-exchange analytical column with excitation at 330 nm and emission at 465 nm. The calibration curve was prepared using glyphosate standard (Monsonto) hydrolyzed with the already-mentioned protocol. Potassium dihydrogen phosphate at 0.005 *M* was used as mobile phase at a flow rate of 1 mL/min.

One unit (U) of glyphosate C-P lyase was defined as the amount of glyphosate C-P lyase that converts 1 μ*M* of glyphosate per minute under the conditions of the assay.

Protein content was measured according to Bradford,[26] using bovine serum albumin (BSA) as a standard.

Purification of Glyphosate C-P Lyase

Precipitation

Crude enzyme filtrate was precipitated by adding different ice-cold solvents like isopropanol, ethanol, acetone, and methanol at a 1:2 ratio with constant stirring for 2 hr at 4°C and the precipitate was separated by centrifugation at 10,000× g for 20 min at 4°C. The precipitate was dissolved in a minimal volume of phosphate buffer (50 m*M*, pH 7.0) and dialyzed against water for 24 hr at 4°C and assayed for enzyme activity.

Ion-Exchange Chromatography

Methanol-precipitated enzyme, which showed highest activity, was passed through a DEAE cellulose cation-exchange column using phosphate buffer (50 m*M*, pH 7.2) for elution. Glyphosate C-P lyase activity-containing fractions obtained after the ion-exchange chromatography were pooled, dialyzed with distilled water, and concentrated with a bench-top lyophilizer.

Size Exclusion Chromatography

The dialyzed and lyophilized sample from the previous step was loaded on 50 m*M* phosphate buffer, pH 7.0, preequilibrated Sepharose 6B column (1.1 cm × 50 cm). The protein elution was done with the same buffer at a flow rate of 0.4 mL/min. Fractions of 5 mL each were collected.

The absorbance at 280 nm was recorded and active fractions were pooled, concentrated, and dialyzed against distilled water. This concentrated fraction was stored at −20°C.

Criteria of Purity

HPLC

Purified enzyme was chromatographed in a Shimadzu high-performance liquid chromatography (HPLC) system with reverse-phase C18 column (Thermohypersil, 300 × 4.6 mm, 5 m ODS) using the following protocol: 1–10 min A (0.1% trifluoroacetic acid [TFA]) 100%, 10–70 min, B (acetonitrile 80% containing 0.05% TFA) 100%, 70–75 min, B 100%, 75–80 min A 100%, 80–90 min A 100%. Detection was carried out at 273 nm.

Polyacrylamide Gel Electrophoresis (PAGE)

The lyophilized C-P lyase enzyme obtained from the Sepharose 6B column was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for ascertaining the homogeneity and to estimate the molecular mass of the protein. SDS-PAGE was performed on a slab gel containing 10% polyacrylamide, which was employed in order to achieve separation of proteins.[27] Alcohol dehydrogenase (150 kD), bovine serum albumin (66 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), lactoglobulin (18 kD), cytochrome *c* (12.4 kD), and aprotinin (6 kD) were used as reference standards.

Proteins were stained with Coomassie brilliant blue R-250 and also silver nitrate. Native PAGE of the purified C-P lyase was performed on 7.5% polyacrylamide gel in glycine buffer at 5 ± 1°C as described by Gallagher.[28]

Effect of pH and Temperature

Glyphosate C-P lyase activity as a function of pH was determined by using 0.1 *M* buffers with pH values of 4.0 to 8.5 for 1 hr. The buffers used were sodium acetate (pH 4.0 to 6.0) and sodium phosphate (pH 6.5–8.5). Effect of temperature was determined by incubating reaction mixture from 20°C to 50°C for 1 hr.

Effect of Inhibitors, Detergents, and Metal Salts

Phenylmethanesulfonyl fluoride or phenylmethylsulfonyl fluoride (PMSF), *N*-bromosuccinimide (NBS), urea, ethylenediamine tetraacetic acid (EDTA), and sodium azide were evaluated at 2 to 5 m*M* levels for their effectiveness as inhibitors on glyphosate C-P lyase activity using glyphosate (2 m*M*) as substrate at optimum pH and temperature for 1 hr. Triton X-100, Tween 20, Tween 80, and SDS were tested for their effect on glyphosate C-P lyase activity at 2% level. Metals including NaCl, KCl, $CoCl_2$, $CuSO_4$, $CuCl_2$, $MgSO_4$, $Pb(CH_3COO)_2 \cdot 3H_2O$, $MnSO_4 \cdot H_2O$, $HgCl_2$, $ZnSO_4 \cdot 7H_2O$, $FeCl_3$, $NaNO_3$, KNO_3, $FeSO_4 \cdot H_2O$, and $MgCl_2$ at 1 m*M* were evaluated

for their effect on glyphosate C-P lyase activity by incubating the enzyme at optimum pH and temperature for 1 hr.

Substrate Specificity

Glyphosate C-P lyase activity was determined with glyphosate, atrazine desethyl, monocrotophos, 2,4-D, parathion, methyl parathion, acephate, glyphosine, dichlorvos, atrazine, simazine, and terbutryn as substrates at a final concentration of 10 m*M*. The relative activity was expressed as the percentage ratio of the enzyme activity determined against different structure analogs of glyphosate to enzyme activity with glyphosate. The rate of the reaction was expressed as specific activity of the enzyme measured in terms residual or unreacted substrate because of enzyme action.

Determination of Kinetic Constants

The kinetic constants, Km and Vmax, of the purified glyphosate C-P lyase were determined by the method of Lineweaver and Burk[29] with different concentrations (0.1–1000 m*M*) of glyphosate as a substrate. Reactions were performed in 50 m*M* sodium phosphate buffer (pH 7) at 30°C.

RESULTS AND DISCUSSION

Glyphosate C-P Lyase Activity

The culture broth of individual isolates obtained from growth medium (NB) did not show any glyphosate C-P lyase activity. However, culture broth of the isolates obtained after induction with glyphosate showed glyphosate C-P lyase activity (Figure 1). Culture broth of *Pseudomonas putida* T5 showed maximum activity. Glyphosate C-P lyase activity of the clarified cell

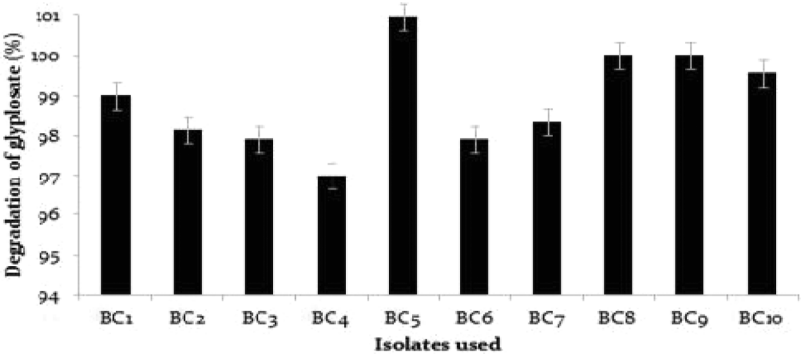


FIGURE 1 Glyphosate degradation in (a) culture broth of bacterial isolates. BC: bacterial culture; BC_1: *Pseudomonas flourescens biovar* I; BC_2: *Pseudomonas diminuta*; BC_3: *Pseudomonas flourescens biovar* II; BC_4: *Burkholderia pseudomallei*; BC_5: *Pseudomonas putida*; BC_6: *Flavobacterium sp.*; BC_7: *Vibrio alginolyticus*; BC_8: *Pseudomonas aeruginosa*; BC_9: *Pseudomonas stutzeri*; BC_{10}: *Pseudomonas flourescens biovar* V.

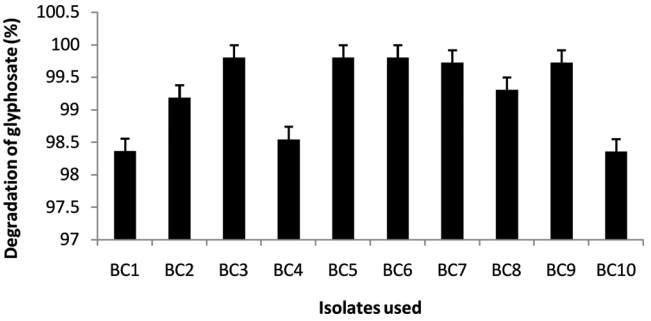


FIGURE 2 Glyphosate degradation in cell-free extracts of bacterial isolates. BC_1-BC_{10} as given in Figure 1.

homogenate (CCH) also designated as cell-free extract (CFE) of individual cells was higher than that of the culture broth (Figure 2). Culture broth of *Pseudomonas putida* T5 showed maximum activity, followed by *Ps. aerugenosa* T8, *Ps. stutzeri* T9, *and Ps. fluorescens* T10 biovar 5. *Burkholderia pseudomallei* T4 showed the minimum activity (Figure 1).

Cell-free extract of *Ps. putida* T5 showed maximum activity, followed by *Ps. fluorescens* biovar 2 T3 and *Flavobacterium* sp. *Vibrio algimolyticus* T7 5 and *Ps. stutzeri* T9 gave good activities. *Pseudomonas fluorescens* T10 biovar 5 showed the lowest enzyme activity (Figure 2). In all the cases enzyme activity in induced cells was higher (9.5-fold) than for uninduced cells (data not shown).

Purification of Glyphosate C-P Lyase Enzyme

The enzyme was purified in three steps. The CFE of *Pseudomonas putida* T5 was precipitated with three solvents, of which methanol was observed to be the best (data not shown). The enzyme precipitate was then passed through a DEAE cellulose column; fractions showing activity were dialyzed, lyophilized, and loaded onto a Sepharose 6B column equilibrated with 50 m*M* phosphate buffer, pH 7.0. The purification steps for glyphosate C-P lyase of *Pseudomonas putida* T5 are given in Table 1. The specific activity of the enzyme increased with every step of purification with a minimum loss in quantity. The purified enzyme after gel filtration step had a specific activity of 176.6 U (Table 1). After every purification procedure, the peak fractions with the enzyme activity were analyzed using SDS-PAGE. The enzyme precipitated out with methanol was observed to have better activity than other precipitants.

TABLE 1
Summary of Steps Employed in Purification of Glyphosate C-P Lyase From *Pseudomonas putida* T5

Purification steps	Total activity (IU)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)
Crude extract	907.27	3240	0.2800	1
Methanol precipitation	1812.04	480	3.7750	13.482
DEAE-cellulose column	5218.42	124	42.084	150.3
Sepharose 6B	6321.50	36	175.60	627.13

Removal of the few contaminating proteins using a DEAE cellulose cation-exchange column was found to be a suitable step. Peak fractions of the DEAE cellulose cation-exchange column were pooled together and loaded onto an equilibrated Sepharose 6B gel filtration column for purification, and in the eluent two protein peaks and one enzyme activity peak were obtained (Figure 3). Peak fractions showing enzyme activity were pooled, dialyzed extensively against distilled water, and lyophilized.

Criteria of Homogeneity

The homogeneity of the purified protein was assessed by PAGE and HPLC (Figures 4 and 5). Activity determinations from native cut gel proteins confirmed glyphosate C-P lyase activity; The dialyzed enzyme was stored in 50 m*M* phosphate buffer (pH 7.5) for 4 months at 4°C with less than 10% loss in activity. Purified enzyme was chromatographed in a Shimadzu HPLC 10AT series with an ultraviolet–visible (UV-vis) variable-wavelength detector (SPD-M10AVP, Shimadzu) using a C-18 reverse-phase column (300 × 6 mm, 5 µm Hypersil) according to Latha et al. (2012).[30] A single peak was observed under the reverse-phase (RP) HPLC conditions (Figure 5).

Molecular Mass Determination by Size Exclusion Chromatography

The glyphosate C-P lyase from *Ps. putida* T5 is an active monomer with an approximate total molecular mass of ~70 kD (Figure 4B). SDS-PAGE revealed only one distinctive band that was indicated by the pure preparation of glyphosate C-P lyase (Figure 4A). SDS-PAGE showed a single band with a molecular mass of ~70 kD. Gel filtration indicated the enzyme with a molecular mass of ~70 kD (Figure 4B). Purified glyphosate C-P lyase was electrophoresed in native gel. A 1.5% agar layer containing 10 m*M* glyphosate showed a clear zone after 24 hr of incubation, indicating the presence of glyphosate C-P lyase (data not shown).

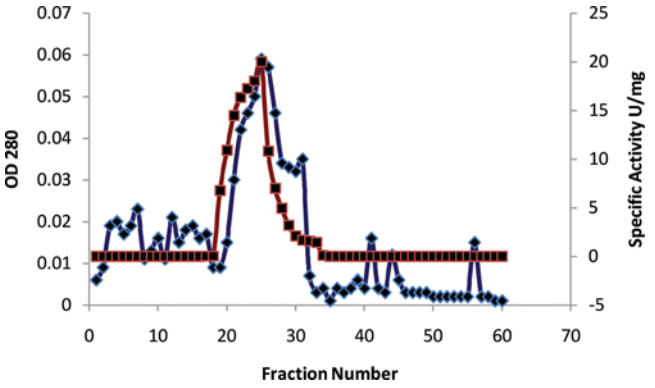
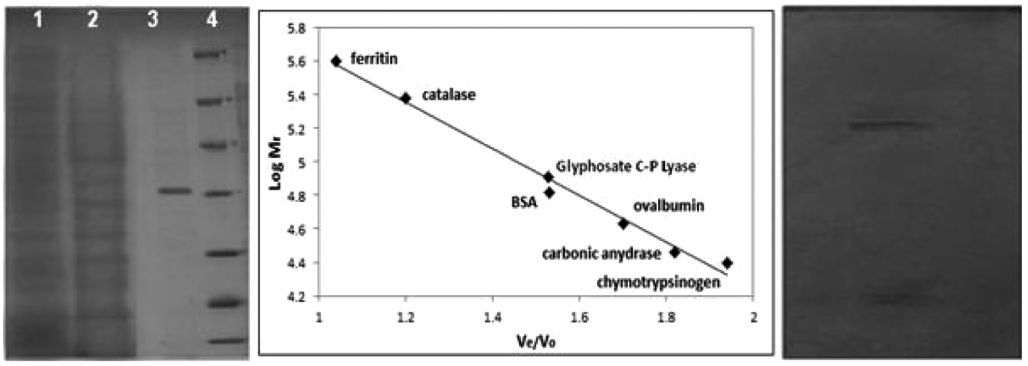


FIGURE 3 Elution profile of gel filtration sepharose-6B column. Blue line indicates protein estimated at 280 nm; red line indicates C-P lyase activity.



(A) (B) (C)

FIGURE 4 Determination of the molecular weight of the purified glyphosate C-P lyase from *Ps. putida* T5. (A) SDS-PAGE was carried out on 10% gel. Lane 1: crude preparation; lane 2: purified glyphosate C-P lyase after DEAE cellulose; lane 3: purified glyphosate C-P lyase after size-exclusion chromatography; Lane 4: standard protein markers of different molecular weights. (B) Gel filtration chromatography using Sepharose 6B column. Standard molecular mass proteins used included chymotrypsinogen (25 kD), carbonic anhydrase (29 kD), ovalbumin (43 kD), BSA (66 kD), catalase (240 kD), and ferritin (398 kD). (C) Native-PAGE of the purified glyphosate C-P lyase from *Ps. putida* T5.

Effect of pH, Temperature, and Stability

Glyphosate C-P lyase from *Ps putida* T5 was active between pH values of 4.0 and 6.0 with an optimum at pH 7.0 (Figure 6). At lower pH the activity was low. It increased slightly with increase in pH and reached a maximum at pH 7.0.

The reaction rate of glyphosate C-P lyase was measured at various temperatures ranging from 4 to 45°C; when the enzyme was incubated at different temperatures for 1 hr and then assayed

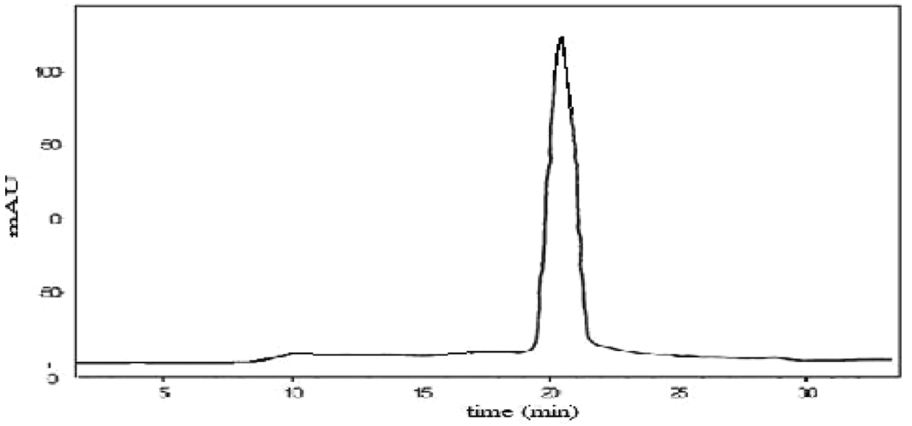


FIGURE 5 HPLC of purified glyphosate C-P lyase from *Ps. putida* T5 on C18 reverse-phase column (300 × 6 mm, 5 μm Hypersil) at 273 nm.

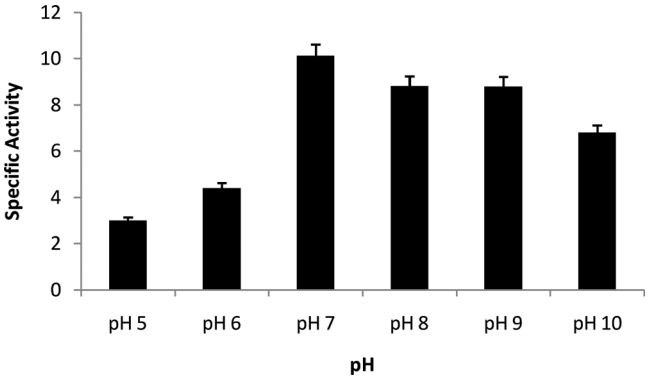


FIGURE 6 Effect of pH on the activity of glyphosate C-P lyase from *Ps. putida* T5. Effect of pH was assayed in the pH range of 5 to 10.

immediately for enzyme activity, there was progressive loss in enzyme activity with increase in temperature (Figure 5). Maximum activity was obtained at ambient temperature of 28 to 30°C. At higher temperatures, the reaction rate declined sharply (Figure 7).

In our studies glyphosate C-P lyase enzyme was stable for 2 hr at optimum pH and temperature (Figures 8 and 9).

Effect of Various Effectors on Glyphosate C-P Lyase Activity

Glyphosate C-P lyase activity was assayed in the presence of different inhibitors/activators (Tables 2 and 3). Cysteine, PMSF, and iodoacetamide showed 63%, 69%, and 73% inhibition, respectively. SDS showed only 2% inhibition, indicating the absences of S-S bridges. However, the inhibition by thiol group blocking reagents, namely, cysteine and iodoacetamide, indicated the role of sulfhydryl groups in the catalytic activity of the enzyme. Inhibition by PMSF

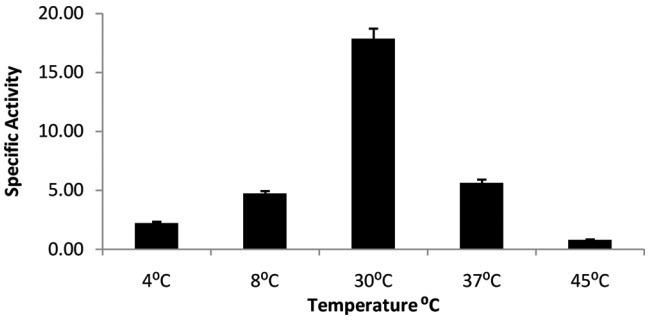


FIGURE 7 Effect of temperature on the enzyme activity. Enzyme activity was assayed at various temperature ranges from 4 to 45°C in 50 m*M* sodium phosphate buffer, pH 7.

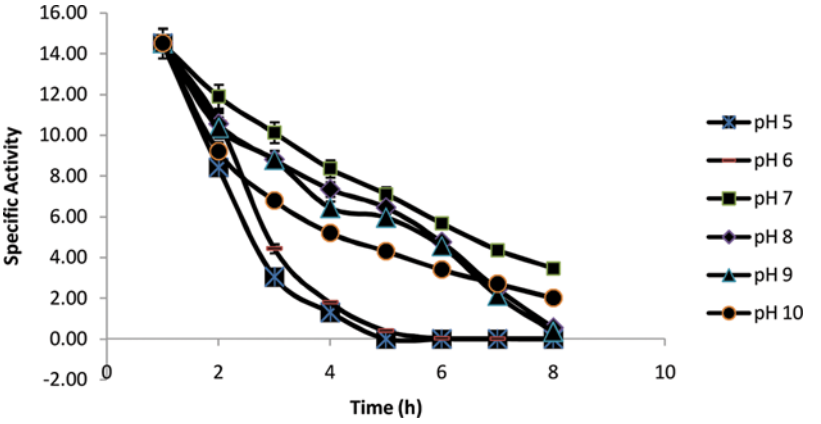


FIGURE 8 pH stability of glyphosate C-P lyase enzyme.

indicated involvement of serine in the active site. Detergents such as Tween 80, Tween 20, and Triton X-100 inhibited the enzyme activity, and there was respectively ~76%, 75%, and 91% inhibition of enzyme activity. The metal chelating agent EDTA did not affect the glyphosate C-P lyase activity, which revealed that the enzyme was not a metalloprotein. Decreased enzyme activity in the presence of NBS (53%) indicated the involvement of tryptophan residues. Urea also showed inhibitory effects (84%) on enzyme activity.

The influence of various metal ions on the purified glyphosate C-P lyase is presented in Table 3. Na^{+} was detrimental to enzymatic activity. Ca^{2+}, Cu^{2+}, K^{+}, and Zn^{2+} inhibited the enzyme activity by 52, 25, 40, and 58%, respectively, whereas Mg^{2+} did not cause inhibition of enzyme activity. The activity was almost retained in the presence of Mg^{2+}. No other metal studied showed enhancement in enzyme activity. Inhibition of enzyme activity in the presence of Cu^{2+} and Zn^{2+} might be indicative of essential vicinal sulfhydryl groups of the enzyme for productive catalysis.

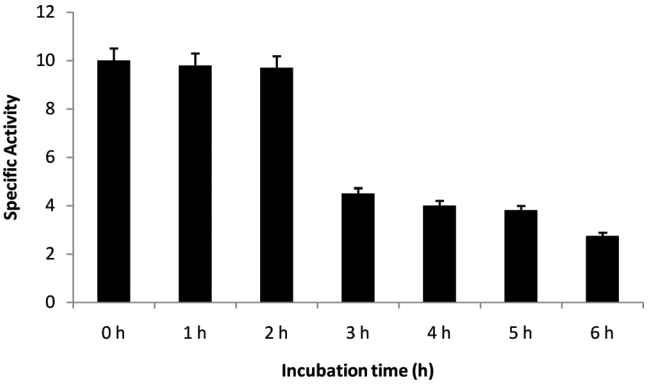


FIGURE 9 Temperature stability of glyphosate C-P lyase enzyme.

TABLE 2
Effect of Inhibitors and/or Activators on Glyphosate C-P Lyase Activity

Inhibitors	Relative activity (%)
Control	100.0
SDS	98.0
EDTA	98.0
NBS	47.1
L-Cysteine	37.4
NAC	35.2
PMSF	31.4
Iodoacetamide	27.3
Tween 20	25.7
Tween 80	24.5
Urea	16.5
Triton X-100	8.4
Glutathione	0.0

Substrate Specificity

Substrate specificity of purified enzyme on various substrates is summarized in Table 4. The purified enzyme had activity against glyphosate and had very low activity against glyphosine. The enzyme was not reactive to any of the other substrates tested, even though some of the substrates had a C-P bond linkage. This property makes glyphosate C-P lyase from *Pseudomonas putida* T5 potentially very useful in remediating glyphosate.

TABLE 3
Effect of Metal Ions on Glyphosate C-P Lyase Activity

Metals	Relative activity (%)
Control	100.0
$MgSO_4$	100.3
$MgCl_2$	99.7
$FeCl_3$	75.5
$CuSO_4$	75.2
KCl	63.9
$BaCl_2$	61.4
KNO_3	60.8
Al_2O_3	59.5
$CaCl_2$	48.7
NH_4Cl	46.6
$ZnSO_4$	42.0
$(NH_4)_2SO_4$	34.1
$Pb(CH_3OO)_2$	22.0
NaCl	0.0

TABLE 4
Effect of Different Substrates on Glyphosate C-P Lyase Activity

Substrate	Specific activity
Glyphosate	65.29
Atrazine desethyl	ND
Monocrotophos	ND
2,4-D	ND
Parathion	ND
Methyl parathion	ND
Acephate	ND
Glyphosine	12.70
Dichlorvos	ND
Atrazine	ND
Simazine	ND
Terbutryn	ND

Note. ND, none detected.

Kinetics Parameters of Purified Enzyme

The enzyme was assayed at varying concentrations of glyphosate (0.1–1000 m*M*). The maximum activity was at 2 mM concentration of glyphosate (Figure 10). This concentration was used throughout the study. Based on an Eadie–Hofstee plot, the Km and Vmax and were calculated as 50 m*M* and 3.42 μ*M*/mL/min, respectively.

Confirmation of the Hydrolytic Mechanism of Glyphosate C-P Lyase

Confirmation of the hydrolytic nature of the carbon–phosphorus bond cleavage activity by purified enzyme was done by evaluating the products formed by LS-MS/MS. Mass spectral analysis showed that a peak corresponding to molecular weight of 89 corresponding to sarcosine was formed as a product of enzyme action on glyphosate. The peak with the molecular weight of ~91 is the result of adduct formed in the LCMS for the compound sarcosine (Figure 11). No other metabolites were observed, indicating the catalysis of glyphosate to sarcosine by glyphosate C-P lyase enzyme.

Compounds with a stable carbon–phosphorus bond are extensively used as antibiotics, herbicides, adhesives, flame retardants, chelating agents, and so on,[31,32] and glyphosate is a herbicide with a stable carbon–phosphorus bond. C-P lyases are of inherent interest because chemistry of the C-P bond cleavage presently is poorly understood.[33] To our knowledge this is the first report on the isolation, purification, and characterization of glyphosate C-P lyase from bacterial sources. Of the 10 isolates screened for glyphosate C-P lyase production, *Pseudomonas putida* T5 showed high enzyme activity. Many bacteria are known to degrade a large number of structurally different C-P compounds. But only a limited range of bacterial isolates has been shown to be capable of the degradation of the widely used broad-spectrum phosphonate herbicide glyphosate [*N*-(phosphonomethyl) glycine]. *Pseudomonas* sp. has been found to be capable of utilizing glyphosate as its sole source of phosphorus. *Pseudomonas* sp. strain PG2982 was shown to carry out the cleavage of the C-P bond, giving rise to the formation of sarcosine.[34]

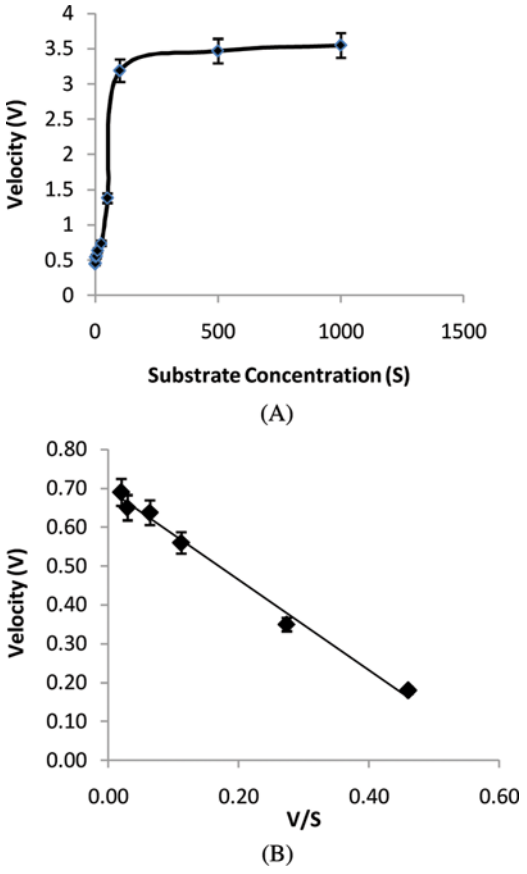


FIGURE 10 Km and Vmax of the purified enzyme. (A) Plot of the reaction velocities (V) versus substrate concentration (S: 0.1–1000 mM) fitted to the Michaelis–Menten equation (maximum activity was at 2 m*M* concentration of glyphosate). (B) The corresponding Eadie–Hofstee plot (Km = 50 m*M* and Vmax = 3.42 μmol/mL/min).

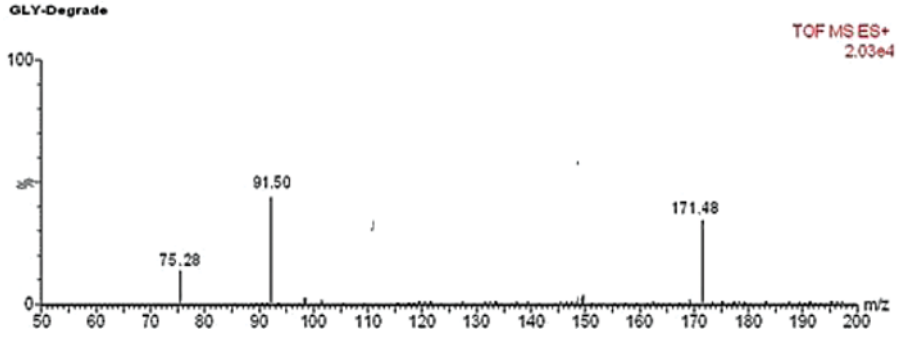


FIGURE 11 LCMS/MS pattern of sarcosine formed as a product of enzyme action on glyphosate.

Essentially the same pathway of glyphosate metabolism was found in *Arthrobacter* sp. strain GLP-1.[35] Although many microbes capable of glyphosate utilization have been isolated from environmental sources,[30] the enzymes of the degradation of the herbicide have not been characterized. Preliminary attempts to demonstrate in vitro activity of either the glyphosate-degrading enzyme or the AMPA-degrading enzyme (C-P lyase) have not met with success.[36] Cell-free degradation of glyphosate in extracts from any of the glyphosate-degrading microorganisms studied so far remains to be demonstrated.[36] *Arthrobacte*r sp. GLP-1 and *Pseudomonas* sp. PG2982 degrade glyphosate by initial cleavage of the C–P bond, resulting in the production of sarcosine (*N*-methylglycine) by C–P lyase activity.[19,37] *Rhizobium meliloti* has also been reported to degrade glyphosate by this pathway, but it had only one C-P lyase, and it was able to degrade a wide range of phosphonates.[38]

The enzyme glyphosate C-P lyase was an intracellular enzyme. However, the culture broth showed very low enzyme activity compared to CCH. The activity in the culture broth could be due to lysis of the cells and release of the enzymes into the culture broth. The CCH of all induced cells showed glyphosate C-P lyase activity. Enzyme activity in uninduced cells was extremely low. That is, induction by glyphosate appeared to be essential for the production of these degradative enzymes. Induction (preexposure) helped in faster degradation of glyphosate without any lag. The enzyme activity of induced cells was higher than for uninduced cells. Since CCH of *Pseudomonas putida T5* showed maximum activity compared to other isolates, it was selected for further studies. Phosphonate monoester hydrolase from *Pseudomonas sp*. PG2982 was cloned into *E. coli* strain CJ236 and the expressed enzyme was purified and characterized.[39]

Glyphosate C-P lyase enzyme of *Pseudomonas putida* T5 could be purified to homogeneity in three steps with phosphonate monoester hydrolase (PEH) in 5% ammonium sulphate and loaded on to a phenyl-Sepharose column equilibrated in 5% ammonium sulfate, 20 m*M* Bis-Tris propane for purification.[39] The SDS and native gel patterns of the CCH of induced cells showed a few extra bands and there were eliminations of a few protein bands present in uninduced cells (data not shown). This would have facilitated easier purification of the protein, as other proteins could have been eliminated during induction. In this study, the purity of enzyme was confirmed by native and SDS-PAGE and HPLC methods. The SDS-PAGE showed the enzyme to be a monomer of ~70 kD mass. The activity of *Pseudomonas putida* was ascribed to the purified enzyme by determination of activity in the region after polyacrylamide gel electrophoresis. The PEH enzyme was composed of 59- and 66-kD polypeptides.[39] Dotson et al.[39] observed PEH activity to be novel because other known phosphonate ester hydrolases were unable to hydrolyze glyceryl glyphosate. Native molecular mass of 240 kD for PEH enzyme (cloned and expressed enzyme) was determined by chromatography on a Sephacryl S400 column. A single polypeptide of 66 kD was observed on SDS-PAGE, confirming the enzyme was a homotetramer. In our studies the molecular mass as determined by SDS-PAGE and size exclusion chromatography was ~70 kD, indicating that the enzyme is a monomer. PEH activity was stable over a pH range of 5–10 at 2- or 12-min preincubation at the various pH conditions. A bell-shaped curve was observed for PEH activity with a maximum activity at pH 9.0.[39] However, glyphosate C-P lyase enzyme (this study) was stable at pH 7.0 for 2 hr, after which the activity decreased. Na^+ was detrimental to glyphosate C-P lyase enzymatic activity. Ca^{2+}, Cu^{2+}, K^+, and Zn^{2+} inhibited the enzyme activity, while Mg^{2+} did not cause inhibition of enzyme activity. However, heavy metal ions such as Cu^{2+} and Zn^{2+} were potent inhibitors. Co^{2+} appeared to stimulate activity of cloned PEH activity. The PEH enzyme has

been shown to hydrolyze both glyceryl glyphosate and β-naphthyl phenylphosphonate.[39] Similarly, glyphosate C-P lyase was found to have high activity against glyphosate and low activity against glyphosine. Thus, the enzyme had narrow substrate specificity and was not acting on other C-P bonds.

The occurrence of this unique enzyme that will hydrolyze a C-P bond of glyphosate may find interesting use in bioremediation of industry effluent or runoff water, as glyphosate is a potent inhibitor of aromatic amino acid biosynthesis. As the enzyme was active at ambient temperature of 28–30°C and pH of 7.0, it could be a good candidate for application in glyphosate bioremediation, reducing glyphosate to a nontoxic, easily degradable metabolite.

Our studies on bacterial degradation of glyphosate showed that glyphosate was metabolised via sarcosine to form glycine.[24] Several mechanisms have been envisaged for this conversion. But in all, the intermediate product of reaction has been shown to be sarcosine as confirmed by both thin-layer chromatography and amino acid analysis. Since the phosphonomethyl carbon is lost as a one-carbon unit at the formaldehyde level of oxidation during the conversion of sarcosine to glycine,[40] no radioactivity from this carbon of glyphosate was incorporated into glycine. Thus, two pathways for the bacterial metabolism of this herbicide with sarcosine as the intermediate during conversion of glyphosate to glycine by *Pseudomonas* PG2982 have been confirmed. Glyphosate was found to be converted to aminomethyl phosphonate in a *Flauobacterium* sp.,[41] as well as in mixed soil cultures of bacteria.[42,43] However, metabolism of glyphosate in *Pseudomonas* PG2982 was not found to involve the intermediate formation of aminomethyl phosphonate. In *Flavobacterium* sp.,[41] conversion of glyphosate to aminomethyl phosphonate occurred in the presence of inorganic phosphate, whereas in *Pseudomonas* PG2982 m glyphosate metabolism to sarcosine was inhibited by inorganic phosphate. *Arthrobacter* sp. GLP-1 also appeared to metabolize glyphosate via the sarcosine pathway[35] and the metabolism was inhibited by inorganic phosphate.

CONCLUSIONS

The occurrence of this unique enzyme that will hydrolyze a C-P bond of glyphosate may find interesting use in bioremediation of industry effluent or runoff water, as glyphosate is a potent inhibitor of aromatic amino acid biosynthesis. As the enzyme was active at ambient temperature of 28–30°C and pH of 7.0, it could be a good candidate for application in glyphosate bioremediation, reducing glyphosate to a nontoxic, easily degradable metabolite.

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