

Biodegradation of 1,1-diphenylethylene and 1,1-diphenylethane by *Pseudomonas putida* PaW 736

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In the present study, the possibility of biodegrading the dechlorinated product of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), 1,1-dichloro-2,2-bis(chlorophenyl)ethylene (DDE) and structurally related compound 1,1-dichloro-2,2-bis(chlorophenyl)ethane (DDD) under aerobic conditions was examined. Since commercial preparation of 1,1-diphenylethane was not available, a structurally related compound, 1,1-diphenylethylene was chosen for the acclimatization of bacterial culture. More than 95% of 1,1-diphenylethylene (initial concentration 500 mg l⁻¹) was degraded by naphthalene degrading *Pseudomonas putida* PaW 736 within six days. GS-MS analysis revealed the formation of benzophenone as the end-product with residual concentration of 157 mg l⁻¹ after six days of incubation. 1,1-diphenylethane was formed from the complete dechlorination of DDT using Mg⁰/Pd⁺⁴ bimetallic system. *P. putida* PaW 736 acclimated to 1,1-diphenylethylene was also able to degrade 1,1-diphenylethane (initial concentration 250 mg l⁻¹) using it as the sole source of carbon and energy. More than 80% degradation of 1,1-diphenylethane was observed following 5 days of incubation. However, any metabolites of the biodegradative pathway for the degradation of 1,1-diphenylethane could not be identified.

Keywords: Biodegradation, 1,1-diphenylethylene, 1,1-diphenylethane, *Pseudomonas putida*.

1,1,1-TRICHLORO-2,2-BIS(4-CHLOROPHENYL)ETHANE (DDT), 1,1-dichloro-2,2-bis(chlorophenyl)ethylene (DDE), and 1,1-dichloro-2,2-bis(chlorophenyl)ethane (DDD) are among the highly persistent and toxic chemicals which were banned in the 1970s by various international regulating agencies. DDT was also designated as a persistent organic pollutant (POP) in 1997 by the governing councils of the UNEP¹. Over the past two decades, there has been intense research around the world to develop methods for the remediation of DDT and other organochlorine pesticides. Currently there is no single method that can address the remediation of DDT under natural environmental conditions. The traditional methods for dechlorination are biological transformation pathways. However, despite the widespread use of DDT for more than 50 years, no

known microbes have yet evolved to mineralize DDT completely and use it as the sole carbon and energy source². Many studies in the literature indicate that various microorganisms isolated from contaminated soil, and wastewater exhibit limited ability to transform DDT into partially chlorinated intermediates like DDD and DDE which are much more recalcitrant³⁻⁵. The main contributing factor to the toxicity of DDT is the presence of chlorine atoms that resist biological degradative reactions⁶. Recently, abiotic reductive dechlorination reactions catalysed by zero-valent metal systems have been applied for the dehalogenation of alkyl and aryl chlorinated compounds. Complete dechlorination of DDT, DDE and DDD into their hydrocarbon skeleton, 1,1-diphenylethane using Mg⁰/Pd⁺⁴ in both aqueous and soil slurry phase was reported earlier⁷⁻⁹. However, there are no reports on the toxic effects, degradation and fate of 1,1-diphenylethane. Hence in the present study an attempt was made to acclimatize a naphthalene degrading *Pseudomonas putida* PaW 736 to 1,1-diphenylethylene (a compound structurally related to 1,1-diphenylethane) and study the biodegradation of 1,1-diphenylethane.

Magnesium (Mg⁰) granules (~20 mesh size), K₂PdCl₆ (hexachloropalladate (IV) dipotassium), DDT (98.2% pure), and 1,1-diphenylethylene (99.5% pure) were purchased from Sigma-Aldrich Chemical Company, USA. Acetone, glacial acetic acid, cyclohexane and other mineral salts were purchased from Merck Ltd (Mumbai, India). No pretreatment was performed with any chemical and they were used as received. All chemicals were of high purity and analytical grade, unless otherwise specified. Technical-grade DDT was procured from Hindustan Insecticide Limited, Maharashtra, India.

1,1-Diphenylethane could not be procured from any chemical company. Hence this compound was obtained through dechlorination of DDT by Mg⁰/Pd⁺⁴ system according to procedure described below⁴. Technical-grade DDT (100 mg) was added into a 250 ml capacity flask from a stock solution prepared in acetone (concentration of stock solution 10,000 mg l⁻¹). Acetone was allowed to evaporate completely. Subsequently, 100 ml of 0.05% JBR biosurfactant solution was added into the flask to dissolve DDT. The reducing catalyst, K₂PdCl₆ was added from a stock solution (stock solution concentration 1000 mg l⁻¹) into the reaction mixtures to obtain 0.1 mg ml⁻¹ final concentration of K₂PdCl₆. Immediately after this, 0.5 g of magnesium granule (Mg⁰) was added to obtain 5 mg ml⁻¹ concentration of Mg⁰. The reaction was initiated by the addition of glacial acetic acid (final concentration of acetic acid in reaction solution was 166 mM). The reaction was conducted at 30°C and atmospheric pressure. Following 48 h of incubation, the reaction mixture was extracted with 300 ml cyclohexane (100 ml, three times), and concentrated to 10 ml under a stream of nitrogen. The concentrate was analysed for the remaining DDT and dechlorinated products by GC-ECD and GC-

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FID analysis. The residual oily product formed from DDT dechlorination was identified as 1,1-diphenylethane using GC-MS (gas chromatography mass spectroscopy).

For biodegradation of 1,1-diphenylethylene and 1,1-diphenylethane, the bacterial strain used was a naphthalene degrading *Pseudomonas putida* PaW 736 procured from Institute of Microbial Technology (IMTECH, Chandigarh, India). First, the bacterial strain was grown on naphthalene (0.1%) as a sole source of carbon. After growth, 1 ml of this culture was transferred to a flask containing 19 ml of mineral medium and 500 mg l⁻¹ of 1,1-diphenylethylene as the sole carbon source. Actual experiments were conducted after three transfers of bacterial strain in 1,1-diphenylethylene-containing medium and this culture grown on 1,1-diphenylethylene was used as inoculum to study the degradation of 1,1-diphenylethylene or 1,1-diphenylethane. Next 500 mg l⁻¹ of 1,1-diphenylethylene and 250 mg l⁻¹ of 1,1-diphenylethane solubilized separately into sterile Tween 80 (0.05% v/v of mineral medium) was added into 19 ml of mineral salts medium as the sole source of carbon and energy. The chemical composition of the mineral medium is given in Table 1 (ref. 10). The pH of medium was 7.1. After adding the bacterial inoculum all the flasks were incubated for selected time periods on a rotatory shaker (set at 150 rpm) at 30°C, following which cultures were sonicated for 5 min using Branson sonifier (set at 5 output and 50% duty cycle) to facilitate the release 1,1-diphenylethylene or 1,1-diphenylethane, if any, absorbed onto the bacterial cells. Two sets of parallel controls were also kept with the test flasks. One set of control was similar to test flask, except that no bacterial inoculum was added to observe abiotic degradation of 1,1-diphenylethylene or 1,1-diphenylethane, if any. Another set of controls were also set up with the bacterial inoculum using only Tween 80 as the sole source of carbon to test the growth of bacterial strain on Tween 80, if any. In this set of control, neither 1,1-diphenylethylene nor 1,1-diphenylethane was added.

After incubation, the entire contents of the flasks were sacrificed, extracted thrice with cyclohexane (total volume 60 ml) and analysed by GC-FID (gas chromatography flame ionization detector) and GC-MS to determine the residual concentrations of diphenylethylene and

diphenylethane, and identify the intermediates/end-products formed respectively.

Bacterial growth was estimated using the dry biomass weight method. The whole culture medium grown either on 500 mg l⁻¹ of 1,1-diphenylethylene or on 500 mg l⁻¹ of 1,1-diphenylethane from each flask was filtered through conditioned (filters were kept in an oven at 105°C for 2.5 h and weighed till constant weight) and pre-weighed filter paper (0.45 µm pore size, Millipore, USA). After filtration, the filter papers were dried in an oven at 105°C for 2.5 h and weighed after cooling them in desiccators. The weight of the biomass was calculated from the pre-weight and post-weight of the filter papers.

All the experiments were conducted in triplicate and standard deviation have been plotted along with average values in the figures.

Analyses of extracted samples were done using an Agilent model 6890 gas chromatographic instrument equipped with FID. The column used was an HP-5 capillary column of 0.32 mm ID, 0.25 µm film thickness and 30 m length. Injection was made in splitless mode using nitrogen as the carrier gas. The following temperature programme was used: the initial oven temperature was 150°C with hold time for 4 min and then ramped at 6°C min⁻¹ up to final temperature of 290°C with hold time for 4 min. The detector temperature was set at 300°C. The residual concentrations of intermediates and end-products were quantified from peak areas obtained through automated integration and also by comparison with known concentrations of pure standard compounds.

GC-MS analyses were performed using Hewlett Packard model G1800A gas chromatographic instrument interfaced with electron ionization detector. A HP-1 capillary column was used with helium as the carrier gas. The column temperature was ramped from 150°C to 250°C at 5°C min⁻¹. Then 70 eV electron impact mass spectra were obtained at the maximum of eluted peaks.

Figure 1 shows an increase in the bacterial biomass concentration from the initial time (immediately after

Table 1. Chemical composition of mineral medium

Constituent	Concentration (g l ⁻¹)
Disodium hydrogen phosphate (Na ₂ HPO ₄ ·12H ₂ O)	9.0
Potassium dihydrogen phosphate (KH ₂ PO ₄)	1.5
Ammonium chloride (NH ₄ Cl)	2.0
Magnesium sulphate (MgSO ₄ ·7H ₂ O)	0.2
Ferric ammonium citrate (Fe(NH ₄) ₃ (C ₆ H ₅ O ₇) ₂)	0.005
Manganese sulphate (MnSO ₄)	0.002
Zinc sulphate (ZnSO ₄ ·7H ₂ O)	0.0002
Cobalt sulphate (CoSO ₄)	10.0
Nitriloacetic acid (N(CH ₂ COOH) ₃)	10.0

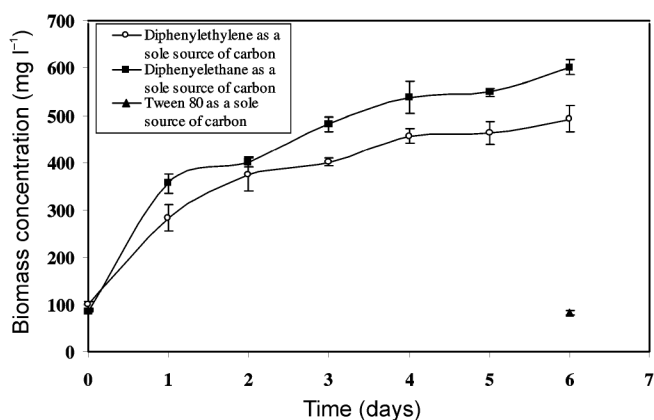


Figure 1. Growth pattern of bacterial culture for the utilization of 1,1-diphenylethylene and 1,1-diphenylethane (initial concentration of both compounds was 500 mg l⁻¹).

inoculation) to day 6 for the utilization of 1,1-diphenylethylene and 1,1-diphenylethane as the sole carbon and energy source. No growth of bacterial culture was observed in the case where Tween 80 was used as the sole source of carbon (Figure 1). The growth pattern of bacterial culture for the utilization of both compounds, i.e. 1,1-diphenylethylene and 1,1-diphenylethane, fol-

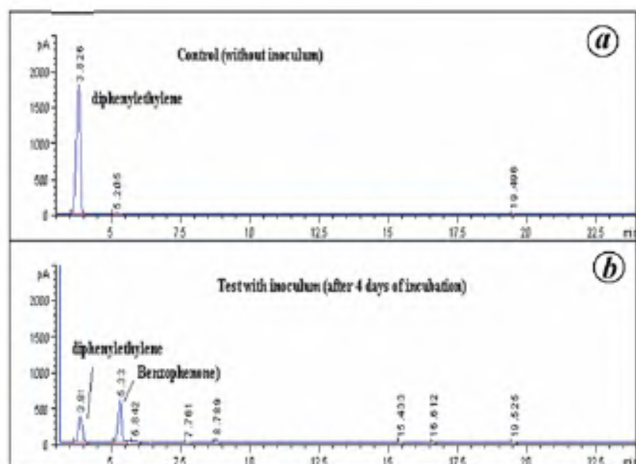


Figure 2. Comparison of GC-FID profiles of extracts derived from (a) control experiment wherein the mineral salt medium containing 500 mg l^{-1} 1,1-diphenylethylene was incubated for 4 days in the absence of microbial inoculum, and (b) test experiments wherein the mineral salt medium containing 500 mg l^{-1} of 1,1-diphenylethylene was incubated for 4 days with the growing bacterial culture under aerobic conditions.

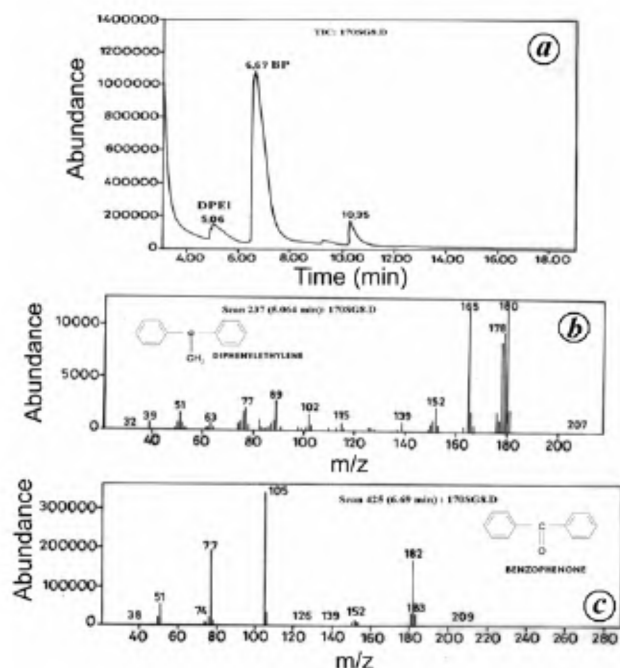


Figure 3. a, GC-MS elution profile of the extract of spent medium following 4 days of growth of *Pseudomonas putida* culture under aerobic conditions using diphenylethylene as the sole carbon source. b, c, GC-MS fragmentation pattern of the peak eluting at (b) 5.064 min and (c) 6.69 min. DPEI, Diphenylethylene; BP, Benzophenone.

lowed the general growth pattern for bacteria. Figure 1 depicts that lag phase for the bacterial growth was less, log phase remained from day 1 to day 4 and the growth was stabilized at the fourth day of incubation. Overall increase in biomass was found higher for the utilization of 1,1-diphenylethane compared to 1,1-diphenylethylene (493.3 and 601 mg l^{-1} respectively, following the sixth day of incubation). Increase in biomass concentration, growth pattern of the bacterial cultures and simultaneous disappearance of the target compounds clearly reveals that the culture utilized the target compounds as the sole source of carbon.

Figure 2 compares the GC-FID profiles for extracts derived from control experiments (wherein 500 mg l^{-1} 1,1-diphenylethylene containing mineral salt medium was incubated for 4 days in the absence of bacterial inoculum) and test experiments (wherein 500 mg l^{-1} 1,1-diphenylethylene containing mineral salt medium was inoculated with bacterial culture and allowed to grow under aerobic conditions for 4 days). Significant reduction in the peak area corresponding to diphenylethylene (retention time of 3.8 min) was noted following incubation with growing bacterial culture. More than 80% of diphenylethylene was biodegraded with the concomitant emergence of a new product peak at 5.33 min. The retention time of this product peak matched that of a standard compound, namely benzophenone. Further, the identity of this product peak was confirmed by GC-MS analysis.

Figure 3a shows the GC-MS elution profile of extracts derived from 1,1-diphenylethylene (500 mg l^{-1}) containing medium following 4 days of incubation with the growing culture of *P. putida* PaW 736 under aerobic conditions. Based on the molecular ion fragmentation pattern, the compound eluting at 5.06 min was identified as 1,1-diphenylethylene (Figure 3b). A major product peak eluting at 6.67 min was identified as benzophenone based on its molecular ion fragmentation pattern (Figure 3c).

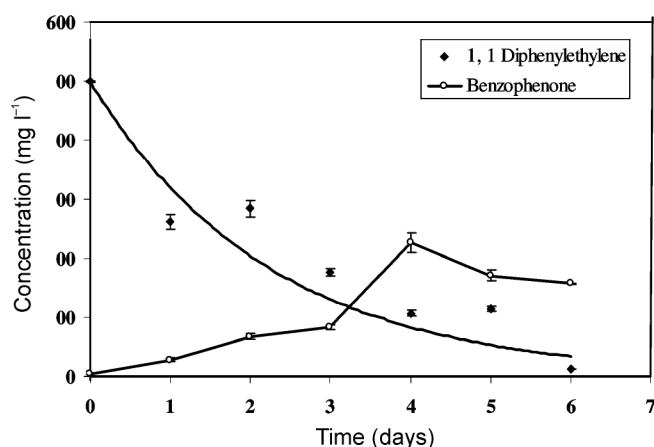


Figure 4. Time course profiles showing degradation of 1,1-diphenylethylene by *P. putida* and concomitant appearance of benzophenone. Diphenylethylene was solubilized in the growth medium using 0.05% Tween 80.

Thus results obtained from GC-FID and GC-MS were in agreement with each other. Time-dependent disappearance of 1,1-diphenylethylene and appearance of benzophenone are shown in Figure 4. Approximately 50% of 500 mg l⁻¹ 1,1-diphenylethylene was degraded following 24 h of incubation, which was accompanied by the accumulation of 27 mg l⁻¹ benzophenone. Following 6 days of incubation, more than 95% degradation of diphenylethylene was achieved. Benzophenone attained highest concentration (226 mg l⁻¹) after 4 days of incubation and then started declining with 157 mg l⁻¹ as residual concentration after 6 days of incubation. To check the disappearance of benzophenone from day 4 to day 6, a similar experiment was conducted as for 1,1-diphenylethane degradation, except that cells were not sonicated prior to extraction. Results supported our earlier report that there was a decline in benzophenone concentration from day 4 to day 6. The possible explanation may be that after day 4, the bacterial culture would have utilized the benzophenone co-metabolically. At day 6 when 1,1-diphenylethylene was growth-limiting, the bacterial culture stopped the metabolizing benzophenone. The appearance of benzophenone indicated that the bacterium was capable of utilizing only one aliphatic carbon presumably through the involvement of a monooxygenase enzyme responsible for the oxidation of alkene (=CH₂) to keto group (=C=O). This result was in accordance with that of Hay and Focht¹¹, who studied the degradation of 100 mg l⁻¹ DDE and its nonchlorinated analogue diphenylethylene (200 mg l⁻¹) by *Pseudomonas acidovorans* M3GY grown on biphenyl. Their result revealed that the bacterium was capable of growing on diphenylethylene using it as the sole source of carbon and energy. Focht and Joseph¹² also reported the degradation of 1,1-diphenylethylene by mixed culture.

An attempt was made to acclimatize *P. putida* PaW 736 for its growth on benzophenone. However, all

attempts to induce the benzophenone biodegradation pathway in the bacterial culture failed.

It may be noted that DPE was not commercially available. Hence all biodegradation studies were carried out using DPE (as a sole source of carbon and energy) generated from reductive dechlorination of DDT by the Mg⁰/Pd⁺⁴ system. Figure 5 compares the GC-FID profiles of extracts derived from the control (wherein 250 mg l⁻¹ 1,1-diphenylethane containing mineral salt medium was incubated for 4 days in the absence of bacterial inoculum) and test experiments (wherein 250 mg l⁻¹ 1,1-diphenylethane containing mineral salt medium was incubated for 4 days in the presence of bacterial inoculum). Approximately 80% of diphenylethane was degraded following 5 days of incubation with the *P. putida* PaW 736. However, metabolites or end-products could not be identified using GC-FID and GC-MS. Absence of benzophenone in the GC-FID profile suggested that the bacterial culture utilized a metabolic pathway which was different from that of the diphenylethylene degradation pathway. Focht and Alexander¹³ isolated a strain of *Hydrogenomonas* that was capable of degrading the structural analogue of DPE, namely diphenylethane with the formation of phenylacetic acid.

The results obtained suggest that highly persistent chlorinated pesticides like DDT, DDD and DDE can be degraded using a sequential chemical-biological approach. As a first step DDT, DDD and DDE can be completely dechlorinated all the way to hydrocarbon end-product, 1,1-diphenylethane using Mg⁰/Pd⁺⁴ bimetallic system. The dechlorinated end-product, 1,1-diphenylethane, and its structural analogue, 1,1-diphenylethylene, can be degraded biologically by *P. putida*. Further research is needed to explore the pathway and enzymes involved in the biodegradation of 1,1-diphenylethane and 1,1-diphenylethylene.

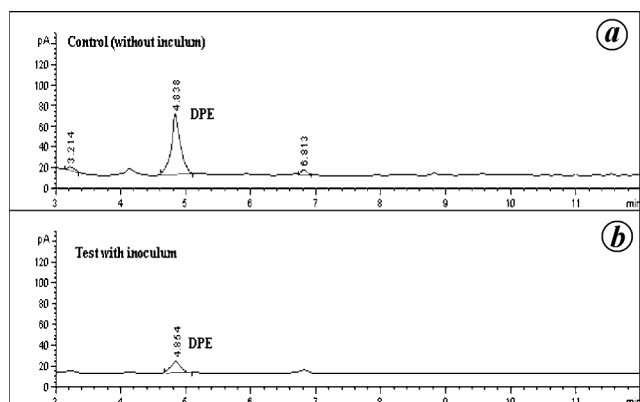


Figure 5. Comparison of GC-FID profiles of extracts derived from the mineral salts medium containing 250 mg l⁻¹ diphenylethane that was incubated for 5 days: (a) in the absence of microbial inoculum and (b) with the growing bacterial culture under aerobic conditions. Diphenylethane (DPE) was solubilized in 0.05% Tween 80 solution.

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Modelling of sPn phases for reliable estimation of focal depths in northeastern India

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In North East India, reliable estimation of earthquake focal depths has always been a problem, owing to paucity of permanent regional seismic stations, constraints on good quality data and lack of crustal models in a complex tectonic terrane. The depth estimations by international agencies, mostly based on teleseismic data, vary from very shallow to even 60 km, suggestive of earthquake occurrence in the upper mantle region. However, modelling of sPn phases in earthquake waveforms that are highly sensitive to focal depths indicates that the earthquake locations are probably well within the crustal layer. The current method has the advantage that dt , the travel time difference between sPn and Pn, remains constant for a wide range of source-station distances, and hence enables easy identification of the sPn phase, while providing direct and accurate estimate of the focal depth. The approach is also insensitive to location errors and variations in crustal models, a useful feature especially while dealing with sparse data with high location errors. In the present study, earthquakes in the magnitude range 3.0–4.0 recorded by a network of nine broadband stations in NE India have been analysed. Consistent dt values at different stations were observed for each event, enabling precise depth estimation mostly within ± 1 or 2 km. In general, depths of 15–20 km in the Shillong plateau region, >20 km in the Sylhet basin region and shallow <10 km in the eastern Himalayan foothills are confirmed, that correlate well with the local tectonics. Further, we propose that the flat characteristics of the dt curve that begin to change for earthquakes below the Moho, can be a potential tool for discriminating between crustal and sub-crustal earthquakes, as well as for delineation of the Moho using dense regional seismic networks in future.

Keywords: Crustal models, earthquakes, focal depth, reliable estimation, sPn phase.

THE North East Indian region is regarded as one of the most seismically active regions of the world, having experienced two great earthquakes of magnitude >8.5 (the 1897 Shillong earthquake and the 1950 Assam earthquake), more than a dozen earthquakes of magnitude >7.0 and several tens of earthquakes of magnitude >6.0 during the last century (Figure 1). Most of the seismicity is a consequence of the stresses generated by the sustained collision of the Indian and Eurasian plates in the Eastern Himalayas to the north and the India–Burma plate boundary along the Arakan Yoma fold belt to the east. The wedge formed by these active plate boundaries comprises the Shillong plateau, believed to be uplifting even at present¹; the Mikir Hills, and the alluvium-covered Assam valley in the Himalayan foredeep that contains one of the largest Tertiary oil fields of India. The seismicity distribution suggests that several lineaments in the NE Indian region are active, the most prominent being Kopili, that separates the Shillong plateau and Mikir Hills, the Bomdila and Sylhet lineaments².

In the past, paucity of high-quality, permanent seismic networks in NE India together with complex lateral variations in the crustal structure has posed severe constraints on precise hypocentral locations, especially the focal depths of earthquakes. Out of the 43 earthquakes of magnitude >5 reported by ISC in the foredeep, Shillong plateau, Mikir Hills and Eastern Himalayan region during the period 1964–2003, 11 seem to be located in the upper mantle, considering an average Moho depth of 40 km estimated from receiver function studies³. Using broadband waveform modelling, Chen and Molnar⁴ have indicated that earthquakes in the Shillong plateau region occur as

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