3.1 Soil

Soil samples were collected from Rajbandh-oil-depot in Rajbandh-Durgapur-Asansol (RDA) industrial belt in West Bengal, India where the use of Trichloroethylene is abundant. Soils were collected by scrapping aside the top 2 to 3 cm and then using trowel to remove soil in the surrounding zone. Soils of each spot were transferred to autoclaved polythene bags, sealed and stored on ice for transport to laboratory. Soils were air dried, sieved (<2mm), stored at 4°C to maintain the indigenous micro flora.

3.2 Materials

Trichloroethylene and chloroform were obtained from Merck India Limited. Pyridine was obtained from Qualigen Fine Chemical, India. All the other chemicals were purchased from Merck India Limited. All the chemicals were of analytical grade and used without further purification.

3.3 Sterilization of media and glassware

All the media used were sterilized by autoclaving at 121°C temperature, 15 psi pressure for 20 minutes. The glassware and other apparatus were sterilized in an oven at 180°C, for an hour. After sterilization, the media and solutions were cooled to room temperature and then stored under refrigeration for their subsequent use. The sterilized glassware were stored separately in an oven at 60°C and cooled to room temperature before their subsequent use.

3.4 Reagent preparation

Mercuric thiocyanate solution:
Mercuric thiocyanate solution was prepared by dissolving 0.3 g of mercuric thiocyanate in 100 ml of 95% ethyl alcohol.

Ferric alum solution:
Ferric alum solution was prepared by dissolving 6 g of ferric ammonium sulfate in 100 ml of 6N nitric acid
3.5 Analytical method

Fujiwara test:

Fujiwara test was performed to detect the presence of free polychlorinated hydrocarbon in the media (143). In the Fujiwara test, 1 ml sample solution was treated with (1ml) pyridine in an alkaline environment (1 ml of 5N NaOH). It was warmed (60°C) for two minutes. The absorbance of the red pyridine phase was then determined at 470 nm (A_{470}) by Systronics Double Beam UV-Vis Spectrophotometer 2203.

Chloride ion assay

Chloride released by bacterial cells into the assay medium was measured by using a spectrophotometric assay (144). 100 ml of standard chloride solution (563.4 μM of KCl solution) was prepared by using Milli-Q water in volumetric flask. 10 ml of 28.17μM, 84.51μM, 140.85μM, 197.19μM, 253.53 μM and a blank without chloride ions was taken in three sets of clean dry test tubes. 1ml of mercuric thiocyanate and 2 ml of ferric alum solution were added and mixed well until an orange color develops. The absorbance was measured at 460 nm (A_{460}) by Systronics Double Beam UV-Vis Spectrophotometer 2203. The net absorbance vs. the chloride concentration of each standard was plotted (Fig 32). The chloride ion concentrations of the unknown samples were determined by comparing the net A_{460} values against the standard curve. The reactions involved are as follows.

\[
2\mathrm{Cl}^- + \mathrm{Hg(CNS)}_2 \rightarrow \mathrm{HgCl}_2 + 2\mathrm{CNS}^-
\]

\[
4\mathrm{Cl}^- + \mathrm{Hg(CNS)}_2 \rightarrow \mathrm{HgCl}_4^- + 2\mathrm{CNS}^-
\]

\[
\mathrm{CNS}^- + \mathrm{Fe}^{+++} \rightarrow \mathrm{Fe(CNS)}^{++}
\]

3.6 Bacterial Enumeration

No. of viable cells of each bead were determined by plate count of disrupted beads. One bead was crushed in 1 ml of distilled water and from that 1 ml was taken to 9 ml of sterile distilled water, thereafter diluting the culture by a factor of 10. This procedure was repeated until the desired dilution was reached. 0.1 ml of a 1:10,000 dilution was poured onto a surface of solidified pre-poured agar plate and then was spread with a bent sterile rod by the standard spread plate method. Next, the plate was incubated at 37°C. Following incubation, the colonies that developed were counted. This procedure was repeated three times and the results were averaged. Final counts of CFU were taken after 24 h. CFU was calculated as:
No. of Colonies x Dilution Factor  
\[
\text{CFU/ml of original sample} = \frac{\text{No. of Colonies x Dilution Factor}}{\text{Inoculum volume}}
\]
The entire process was performed aseptically in the laminar airflow chamber to avoid chances of contamination.

3.7 Isolation of microorganisms from soil by use of trichloroethylene-enriched medium

Soil samples (5 g each) added to sterile distilled water (50 ml) in an Erlenmeyer flask (250 ml) separately was incubated on a rotary shaker (90 rpm) at 37°C for 24 h. The supernatant was used as inoculum (5% vol/vol) to a synthetic TCE containing medium (100 ml), and it was incubated at 37°C for 24 h in a culture with shaking. The synthetic medium contained (g l⁻¹): Peptone, 5; Beef extract, 3; Sodium chloride, 5; trichloroethylene, 1μl/ml; pH 6.9. Samples showing good growth were further enriched by transfer to fresh medium. Colonies were isolated by the dilution plate technique. They were maintained on agar medium I, which contained (g l⁻¹) peptone, 2; Beef extract, 3; Sodium chloride, 5; agar, 1.8; trichloroethylene, 1μl/ml; pH 7.2.

3.8 Screening of trichloroethylene degrading strain with the help of minimal broth containing trichloroethylene as the sole carbon source

The colonies to be screened for trichloroethylene degrading activity were grown at 37°C for 72 h in a modified M9 liquid medium I contained (g l⁻¹) Na₂HPO₄, 6; NaCl, 0.5; KH₂PO₄, 3; NH₄Cl, 1; MgSO₄·7H₂O, 0.5; trichloroethylene, 0.2 μl/ml; pH 7.2. Trichloroethylene was added at 24 h interval for three days. Fujiwara test was performed to detect of free polychlorinated hydrocarbon in the media at the beginning and after two days. Trichloroethylene degrading colony was transferred to nutrient agar slant for further study.

3.9 Characterization and identification of the isolated strain (2479)

Taxonomic characterization of the isolated strain (2479) was done according to Classic Dichotomous Key for Clinically Important Genera (145). The carbon source
utilization pattern was studied with BIOLOG Microlog system using GP2 96 well plate system which was available at Central Inland Fisheries Research Institute, Barrackpore, West Bengal, India in order to identify the novel bacterium. The novel isolate was also sent to Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Sector 39-A, Chandigarh, India for identification. Fatty acid methyl ester test were also perform to identify the novel isolate at IMTECH.

3.10 Scanning Electron Microscope (SEM) of 2479 and beads of 2479 immobilized in ca-alginate and agar-agar

The bacterium was studied using Hitachi, S530 SEM at University Science Instrument Centre, Burdwan University (Fig 5). The bacterium was fixed with glutaraldehyde (4% vol/vol) to stabilize the cell structure. The specimen was dehydrated with acetone. Complete dehydration was essential. The specimen was soaked in unpolymerized, liquid epoxy plastic until it was completely permeated and plastic was hardened to form a solid block. Thin sections were cut from this block with a diamond knife using a special instrument called ultra microtome. Metal coating with gold vapour was carried out. The alginate beads were also studied under SEM (Fig 6 and Fig 7). The specimen preparation was same as described above.

3.11 Optimization of medium

The modified M9 liquid medium was further modified to optimize different cultural parameters for the growth of Bacillus cereus 2479 and degradation of TCE. The exponentially growing cells were added in the culture flask containing 100 ml modified M9 liquid medium I. The medium contained (g l⁻¹) Na₂HPO₄, 6; NaCl, 0.5; KH₂PO₄, 3; NH₄Cl, 1; MgSO₄. 7H₂O, 0.5; TCE, 2 µl/ml, pH 7.2. Growth of the cells in the modified M9 liquid medium-I was measured turbidometrically by measuring absorbance at 540 nm (A₅₄₀). Growth of 2479 was studied for varying different components of the medium. Degradation activity was also studied for varying different components of the medium. Degradation was also studied by Fujiwara test. The absorbance of the aqueous phase was then determined at 470 nm (A₄₇₀) by Systronic Double Beam Spectrophotometer2203.
3.12 Immobilization of cells of *Bacillus cereus* 2479 for trichloroethylene egradation

Cells (200mg wet wt) suspended in Tris-HCl buffer (10ml, 0.05M, pH 8.0) were mixed thoroughly with sodium alginate solution (15ml, 3.3% wt/vol). The resultant suspension was dropped into a magnetically stirred solution of 0.1 M CaCl₂ to obtain spherical beads. The beads were stirred further for 1h, washed, and stored at 4º C. In case of immobilization in agar-agar, cells (200mg wet wt) were mixed with molten agar (300mg). Beads were formed by dropping the mixture into cold solution of KCl (1M) and liquid paraffin. The gel beads were washed and stored at 4º C for future use.

3.13 Determination of trichloroethylene degrading activity of immobilized cells of 2479

Immobilized cells (0.5g) were incubated with trichloroethylene in Tris-HCl buffer (0.05M, pH 8.0, 20 ml) at 37ºC for 24 h. After the incubation was done, beads were removed by filtration. The filtrate was then centrifuged at 8,000 g for 10 min to remove the fine particles. The clear supernatant was then used for Fujiwara test.

3.14 Retention of trichloroethylene degrading activity by immobilized cells

Cells (100mg wet wt) immobilized on different matrices and an equivalent amount of free cells were incubated separately with trichloroethylene in Tris-HCl buffer (0.05M, pH 8.0, 20 ml) at 37º C for 24h. Fujiwara test was performed to estimate the concentration of free polychlorinated hydrocarbon in the media at the beginning and after 24 h.

3.15 Preparation of preinduced inocula for chloroform degradation

The selective trichloroethylene- enriched medium has been used for the preparation of inoculum. The composition of the medium was as follows (g l⁻¹): NH₄Cl, 3; KH₂PO₄, 0.5; CaCl₂, 1.0 and MgSO₄, 0.5; peptone, 2 and trichloroethylene, 0.2 μl/ml. The pH of the media was adjusted to 7.2. The trichloroethylene- enriched medium was inoculated with a loopful of culture of *Bacillus cereus* 2479 and incubated at 30ºC in a shaker (160 rpm) and grown for 24h. This preinduced inoculum was used for further study.
3.16 Stock solution for chloroform

Stock aqueous solution of CF was maintained in 250-ml glass bottle with Teflon-lined rubber septa and aluminum crimp tops. A 120-ml aliquot of Milli-Q water was added to approximately 10 ml of CF with glass beads added to promote mixing. A syringe was used to remove saturated aqueous solution and care was taken to exclude non-aqueous-phase CF.

40 µM chloroform was added as a diluted aqueous solution from the stock which was made freshly at room temperature (approx. 21°C). The concentration of chloroform (assuming that all of the chloroform was in aqueous phase) was calculated using dimensionless Henry’s law constants (146).

3.17 Calculation of 40 µM solution of Chloroform

1 ml chloroform was mixed with 20 ml water in a 100 ml conical flask at 21 °C.

1. Molecular weight of chloroform is 119.38 gmol⁻¹
2. Density of chloroform is 1.487 g ml⁻¹
3. Solubility of Chloroform in Water is 0.8 g/ 100 ml
4. Dimensionless Henry’s law constant is 0.11

2ml aqueous phase was taken.

From Henry’s Law we have p = K_H/X_2 where K_H is the Henry’s Law Constant and X_2 is the mole fraction of solute (Chloroform). We have, K_H = C_1/C_2 where C_1 and C_2 are the concentration of Chloroform in Liquid and Gas Phase respectively.

Therefore, total amount of chloroform dissolved in 1 ml of water is = (0.8 g x 1 ml)/ 100 ml = .008 g

Hence, total number of moles of chloroform in 1 ml water is = 0.008 g/119.38 g mol⁻¹ = 6.7013 x 10⁻⁵ moles

Now, if 21 ml of a concentrated solution of chloroform in water is taken in a 100 ml conical flask and sealed and allowed to come to equilibrium with its vapor phase then,

Let No. of moles of chloroform remaining in solution be = x

Therefore, moles of chloroform in vapor phase = (6.7013 x 10⁻⁵ - x )

Therefore Conc. C_1 = x/21 moles ml⁻¹
And Conc. C2 = (6.7013 x 10⁻⁵ - x )/79

K_H = C_1/C_2
0.11 = (x/21) / (6.7013 \times 10^{-5} - x) / 79
Therefore, x = 1.904 \times 10^{-6} \text{ moles}
Therefore Concentration of the solution is = ((1.904 \times 10^{-6} \text{ moles}) \times (1000 \text{ ml} l^{-1}))/21 \text{ ml}) = 9.066 \times 10^{-5} \text{ M}
2 \text{ ml of this was diluted to 100 ml, hence Conc. Of the stock solution =}
2 \text{ ml} \times 9.066 \times 10^{-5} \text{ M} = 100 \text{ ml} \times Y \text{ M}
Y = (2 \text{ ml} \times 9.066 \times 10^{-5} \text{ M})/ 100 \text{ ml} = 1.813 \times 10^{-6} \text{ M} = 1.813 \mu \text{M}.

If 100 ml of 40 \mu \text{M solution is required then:}
(100 \text{ ml} \times 40 \times 10^{-6} \text{M})/ (9.066 \times 10^{-5} \text{ M}) = 4.41 \text{ ml}

3.18 Culture condition and preparation of cell suspension for chloroform degradation

The strain 2479 was grown at 30^\circ \text{C} for 48 h in modified liquid M9 medium II containing (g l^{-1}) Na_2HPO_4, 6; NaNO_3, 0.5; KH_2PO_4, 3; (NH_4)_2SO_4, 1; MgSO_4. 7H_2O, 0.5; chloroform 40 \mu \text{M}; pH 7.2 on a rotary shaker at 175 rpm. The biological destruction of CF by the strain of 2479 was analyzed by Fujiwara test and chloride ion analysis.

3.19 Growth measurement and Culture density determination for CF degradation

Growth of the cells in the modified M9 liquid medium-II was measured turbidometrically by measuring absorbance at 540 nm (A_{540}). The modified M9 liquid medium-II contained (g l^{-1}) Na_2HPO_4, 6; NaNO_3, 0.5; KH_2PO_4, 3; (NH_4)_2SO_4, 1; MgSO_4. 7H_2O, 0.5; CF, 40 \mu \text{M}; pH 7.2. Systronics Digital pH Meter 335 was used to adjust the pH of the culture media in all cases. Culture density was determined by measuring the mass differences between pre-weighed aluminum foil dishes with 20 ml of liquid medium added and those with 20ml of cell culture added after both sets were incubated overnight at 105^\circ \text{C}.

3.20 Immobilization of cells of Bacillus cereus 2479 for chloroform degradation

Sodium alginate, 5-30 g l^{-1} were used for cell immobilization. The pre-induced bacterial cells (200 mg) were re-suspended in Tris-HCl buffer (10ml, 0.05M, pH 8.0). The mixture was dropped into 100mM calcium chloride (CaCl_2) using a peristaltic pump to obtain equal size beads (3mm). The resultant gel beads were hardened by re-suspending into a fresh CaCl_2 solution for 24 h at 4^\circ \text{C}. Finally, these beads were washed with distilled
water to remove excess calcium ions and unentrapped cells and stored at 4 °C for future use. All the experiments with immobilized cells have been performed in triplicate and data presented as mean ±SE. The entire process was performed aseptically in the laminar airflow chamber.

### 3.2.1 Repeated batch cultivation

Repeated batch fermentation was carried out by decanting the spent medium every 48 h and replacing it by a fresh chloroform containing modified liquid M9 medium II (as mentioned earlier), after washing the alginate beads with sterile distilled water. The decanted spent medium was used for Fujiwara test and chloride ion estimation. Each fermentation cycle was carried out in a 250 ml Erlenmeyer flask containing 100 ml medium at 30°C and 170 rpm shaking.