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Original Article

Isolation and Protein Characterization of Lindane Degrading Root Epiphytic Bacterium *Arthrobacter* sp. T16 from *Typha latifolia*

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Abstract

Lindane, extensively used as pesticide, causes severe environmental hazard and is a threat to the humanity. The present study aims to assess the capability and mechanism of root epiphytic bacteria of wetland plant *Typha latifolia* to degrade lindane. Isolation of lindane degrading root epiphytic bacteria was done by standard enrichment technique and lindane degradation analysis was done using Gas Liquid Chromatography. Bacterial strain *Arthrobacter* sp. T16 was isolated and identified, which showed maximum degradation of 71.2 ± 1.3% of 50 mg l^{-1} lindane. Lindane biodegradation was accompanied with decrease in pH, increase in chloride ions concentration of culture medium and a positive dechlorination assay. Biodegradation potential of *Arthrobacter* sp. T16 was also studied at different lindane concentrations. Maximum degradation was observed at 10 mg l^{-1} lindane followed by 50 mg l^{-1} and 100 mg l^{-1} lindane. Lindane biodegradation kinetics study inferred that the average rate of lindane degradation increased with increase in lindane concentration. Lindane induced proteins in *Arthrobacter* sp. T16 were studied by SDS-PAGE. Distinctive polypeptides came into view in the presence of lindane and were identified as putative ABC transporter periplasmic amino acid-binding protein, elongation factor Tu and trifunctional transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase, each expressed due to lindane stress. This study specifies the potential of phytoremediation in controlling the environmental contamination problem with the help of indigenous organisms present in roots of plants.

Keywords: accession number; bacterial suspension; half-life; mesocosm; pellet

Introduction

Lindane (γ-HCH) is a chlorinated saturated hydrocarbon, extensively used as pesticide. Owing to its chemical properties, it persists in the environment, migrates over long distances with air, undergoes volatilization in tropical conditions, and gets deposited in colder regions, thus causing widespread contamination. Lindane residues also reach animals and humans by food chain and get bioamplified at each trophic level (Benimeli *et al*., 2008; Fuentes *et al*., 2010a; Jennings and Li, 2014; Polti *et al*., 2014). Its residues are found in soil, sediments, water, plants, animal tissues, and even in bodily fluids like breast milk, blood, and amniotic fluid (Vijgen *et al*., 2011; Alvarez *et al*., 2012a). It is classified as possible carcinogen as well as endocrine disruptor, accompanying confirmed mutagenicity, teratogenicity, and genotoxicity (Kaur *et al*., 2013). In 2006, more than 50 countries have taken up the convention on prohibiting or restricting the production and use of lindane. In 2009, lindane was incorporated among persistent organic pollutants (POPs) that cause severe environmental hazard (Draft Risk Management Evaluation for Lindane, 2007; Egorova *et al*., 2017). Though, its use has been disallowed or sternly limited in about 52 countries but it is still being used by some developing countries on economic grounds. The environmental contamination with lindane is a threat to the humanity. Therefore there is an urgent need for the advancement in technologies aiming to decontaminate lindane polluted sites.

Phytoremediation is a strategy to remediate organic contaminants by plant–microrganism interactions or numerous plant methods (Gerhardt *et al*., 2009; Alvarez *et al*., 2012b). Plants, in union with their rhizospheric, root epiphytic (bacteria present on the surface of roots) or endophytic microorganisms, have immense potential to degrade organic pollutants (Abhilash *et al*., 2011; Khan *et al*., 2013; Singh and Singh, 2017). Till date a number of studies have reported bioremediation of lindane by aquatic and soil microorganisms (Benimeli *et al*., 2007; Lal *et al*., 2010; Alvarez *et al*., 2012; Giri *et al*., 2014) but very little work has been done on bioremediation of lindane by root epiphytic or rhizospheric bacteria. Böltner *et al*. (2008) have studied HCH degrading *Sphingomonas* strains colonizing the roots of *Zea mays*. *Sphingomonas* sp., *Devosia* sp.,

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Pseudomonas sp., and *Sphingobium* sp. possessing the ability to degrade HCH were isolated from the rhizosphere of *Phragmites australis* (Miguel *et al*., 2014).

Since organochlorine pesticides are toxic to a number of plant species, therefore the plants selected to be used in phytoremediation must flourish in pesticide polluted sites (Perez *et al*., 2008). In this study, we have chosen *Typha latifolia* for phytoremediation of lindane. *Typha latifolia* (broadleaf cattail) is a wetland species having high level of biomass, high growth rate, and well-developed root system and is well recognized in mitigating environmental pollution (Sukumaran, 2013; Strungaru *et al*., 2015). The present study attempts to assess the capability and mechanism of root epiphytic bacteria of *Typha latifolia* to degrade lindane. So far as known, this is the first report studying the isolation of lindane degrading bacteria from the root surface of *Typha latifolia* and characterizing the unique proteins expressed in the presence of lindane. The objectives of the study were to identify lindane degrading root surface bacteria of *Typha latifolia*, to study the degradation kinetics and lindane induced modification in protein profile of isolated bacteria.

Materials and Methods

Chemicals and culture media

Lindane (> 99 % pure, purchased from Sigma Aldrich, USA) stock was prepared in ethyl acetate at the concentration of 5 mg ml^{-1}, sterilized by filtering and added to the culture media. For bacterial isolation and degradation experiments, we used Mineral Salt Medium (MSM: Diammonium hydrogen orthophosphate, 0.5 g l^{-1}; Magnesium sulphate, 0.2 g l^{-1}; Ferrous sulphate heptahydrate, 0.01 g l^{-1}; Dipotassium hydrogen orthophosphate, 0.1 g l^{-1}; Calcium nitrate tetrahydrate, 0.01 g l^{-1} pH 7.0) and for the growth of isolated cultures, LB medium (Tryptone 10 g l^{-1}; Yeast extract, 5 g l^{-1}; Sodium chloride, 5 g l^{-1}; Glucose 1 g l^{-1} pH 7.0). All the chemicals were of analytical grade and purchased from standard manufacturers.

Root sampling

The root samples were collected from established wetland mesocosms maintained at IARI, Delhi (28° 38' 21.3" N and 77° 08' 56.5" E). IARI campus is traversed by a network of sewage drains that receive domestic and industrial effluents generated by the campus as well as commercial, agricultural, industrial, and dwelling units around IARI. The experimental mesocosms are planted with *Typha latifolia* and flooded with wastewater. Roots sections of about 15 cm were carefully cut from the plants in a way as not to destroy the plants. Roots from three to five individual plants were combined to form sufficient sample for study. The samples were collected in sterile sampling bags. They were taken to lab in containers filled with ice where temperature inside did not exceed 7 °C. Samples were stored at 4 °C in the lab and were analyzed the same day.

Isolation of lindane degrading root epiphytic bacteria

Isolation of root epiphytic bacteria was carried out by standard enrichment technique. Roots were gently washed twice in sterile distilled water and aseptically chopped into small pieces. Root surface bacteria were isolated by shaking small pieces of roots on a mechanical shaker in phosphate buffer saline (1X) at 150 rpm for 1 h. 5 ml bacterial suspension was inoculated in 45 ml MSM containing 50 mg l^{-1} lindane and incubated for seven days at 30 °C on rotary shaker. Enrichment was carried out for a period of five weeks. Spread plate and streaking method were used to obtain pure isolates.

Lindane degradation in liquid culture and identification of strain

The bacterial isolates showing luxurious growth on MSM (with lindane) agar plates were subjected for degradation study. Lindane degradation study was conducted in 100 ml MSM supplemented with 50 mg l^{-1} lindane. Bacterial pellets of 1 ml culture grown in LB were used to inoculate experimental flasks. Controls flasks spiked with lindane were uninoculated. Degradation experiment was set in microbial shaker at 30 °C with 150 rpm for 15 days. Analysis of lindane degradation was done using Gas Liquid Chromatography (Agilent 7890A) equipped with 63 Ni Electron capture Detector (ECD) and HP 05 column. The GC oven temperature was programmed for an initial temperature of 150 °C then hold for 2 min, ramped at a rate of 5°C min^{-1} till180 °C and hold for 3 min and finally ramped at a rate of 10°C min^{-1} to 210 °C and hold at 210 °C for 5 min. The injector and interface temperatures were 220 °C and 250 °C, respectively. Nitrogen was used as carrier gas. 1 µl of sample was injected in split mode (pulse pressure = 12 psi, split ratio 100:1 and purge flow 3ml min^{-1}). Obtained peak area and their retention time (R_t) were used to study lindane degradation. $OD_{\lambda 595nm}$ and pH change during degradation were also studied.

The bacterial strain showing maximum lindane degradation was sequenced from SciGenome labs, India after 16S rRNA gene amplification. The 16S rRNA gene sequence was deposited in GenBank under accession number MF109974 and subjected to BLAST, using National Center for Biotechnology Information (NCBI)/Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The biochemical characteristics of the isolated strains are presented in Table 1.

Dechlorination assay

Dechlorination assay of lindane was performed by the method described by Phillips *et al*. (2001). In a 96-well microtitre plate, three types of wells were made. First set contained assay buffer (Hepes buffer, Sodium sulphate and Na_2EDTA, pH 8), second set had assay buffer and cell free extract of bacteria, third treatment had assay buffer, cell free extract of bacteria and substrate (lindane 10 mg l^{-1}, 50 mg l^{-1} and 100 mg l^{-1}). The microtitre plate was covered and incubated for 6-8 h at 30 °C. Change in color from red to yellow was observed.

Determination of chloride ion concentration was done according to the method described by Iwasaki *et al*. (1952). For this, bacterial strain was cultured in MSM supplemented with 50 mg l^{-1} lindane. 1ml sample was treated with mercuric thiocynate solution and ferric alum solution. The colour developed was measured at λ_{460nm} by spectrophotometer. The amount of Cl^- release was calculated using a standard curve prepared using KCl.

Table 1. Biochemical characteristics of *Arthrobacter* sp. strain T16

Gram staining	✓
ONPG	-
Lysine utilization	✓
Ornithine utilization	✓
Urease	✓
Phenylalanine Deamination	-
Nitrate reduction	✓
H_2S production	-
Citrate utilization	-
Voges Proskauer's	-
Methyl red	-
Indole	-
Malonate utilization	-
Esculin hydrolysis	-
Arabinose	-
Xylose	-
Adonitol	-
Rhamnose	-
Cellobiose	-
Melibiose	-
Saccharose	-
Raffinose	-
Trehalose	-
Glucose	✓
Lactose	-
Oxidase	-
Catalase	✓

✓= Positive - = Negative

Lindane degradation kinetics study

MSM was supplemented with different lindane concentration (10 mg l^{-1}, 50 mg l^{-1} and 100 mg l^{-1}) and inoculated with bacterial strain grown in LB medium. Extraction and degradation study of lindane were done as described previously. Average rate of lindane degradation at its different concentration was calculated using the following equation.

$r_{av}(mgl^{-1}d^{-1}) = C_o\text{-}C_t/(\Delta t)$

where Co is concentration of lindane at time 0, Ct is concentration at time t, r_{av} is the average rate of degradation and Δt is the duration of degradation study.

Lindane degradation kinetics was fitted using first-order kinetic equation which follows:

$C_t = C_o\text{-}e^{kt}$

where C_o - initial concentration of lindane (at time 0), C_t -concentration of lindane at time t, K is the degradation constant and t is reaction time.

The half-life ($t_{1/2}$) of lindane biodegradation was calculated using the formula:

$t_{1/2} = \ln 2/k$

Protein profile of lindane degrading bacterial strain

Protein profile of lindane degrading bacterial strain was studied. For this, bacteria were cultured under two different conditions: (i) with glucose as carbon source (without lindane) and (ii) lindane as sole source of carbon. The bacterial cells were harvested aseptically from culture by centrifugation (8000 x g for 10 min at 4 °C). Obtained bacterial pellet was washed twice with sterile phosphate buffer saline (0.05 M, pH 6.8), subsequently pellet was dissolved in lysis buffer (Singh and Singh, 2014) and boiled in water bath for 10 min. Lysate was cooled to room temperature and sonicated (nine pulses of 5 s and 5 s cooling) at amplitude of 9 Hz. Concentration of protein in cell lysate was determined by Bradford method. Electrophoresis was done on discontinuous polyacrylamide gel (5% stacking and 10% separating gel) with 50 µg protein/lane at 70 V (through stacking gel), further increasing voltage to 120 V (through resolving gel). Coomassie Brilliant Blue G-250 was used as staining dye for proteins. Gel was visualized on gel doc. Polypeptide bands were eluted using ammonium bicarbonate and 50% acetonitrile solvent (Singh and Singh, 2014). MALDI-TOF MS/MS analysis was done at AIRF JNU, New Delhi

Statistical analysis

The mean of triplicates was analyzed using analysis of variance (ANOVA) with Graphpad Prism 7 software. P values less than 0.05 were considered significant.

Results and Discussion

Lindane degradation and identification of bacteria

In the present study we have isolated lindane degrading root epiphytic bacteria from wetland plant *Typha latifolia* by enrichment culture method. Eight morphologically distinct bacterial colonies capable of showing good growth on MSM agar plates (with lindane) were obtained using streak plate technique. Out of all bacterial strains, strain T16 showed maximum degradation potential of 71.2 ± 1.3% of 50 mg l^{-1} lindane after 15 days in MSM broth at 30 °C (Fig. 1, 2a). In uninoculated flasks (control), lindane decreased by 5.09 ± 0.16% (Fig. 2a) which can be attributed to abiotic factors. Analysis and NCBI BLAST result of the 16S rRNA sequence of the isolated strain T16 showed similarity with genus *Arthrobacter*, therefore the strains was identified as *Arthrobacter* sp. strain T16. Earlier studies have also reported *Arthrobacter* sp. (Datta *et al*., 2000; De Paolis *et al*., 2013) to be capable of degrading hexachlorocyclohexane. Lindane degradation by *Arthrobacter* sp. strain T16 was accompanied by its growth in MSM (Fig. 2a), showing the utilization of lindane as sole carbon source. Sagar and Singh (2011) have also observed the growth of *Fusarium* sp. with lindane as sole source of carbon.

Dechlorination assay

Dehalogenation i.e. the removal of halogen atom from organic halogen compounds is the main reaction in microbial degradation of such compounds. Halogen atoms are usually responsible for the toxicity of these xenobiotic compounds and their elimination decreases defiance of these compounds towards biodegradation. In dehalogenation, halogen atoms are substituted by hydrogen or hydroxyl group (Camacho-Pérez *et al*., 2012).

In lindane degradation experiment of our study, pH of culture medium decreased significantly ($p < 0.05$) during incubation period. However in uninoculated flasks

(control), no significant change ($p > 0.05$) in pH was observed (Fig. 2b). To find out that decrease in pH is due to dechlorination of lindane, dechlorination assay was done. A change in coloration of reaction mixture from red to yellow was observed after 6 hrs of incubation in wells containing assay buffer, cell free extract of bacteria and substrate (lindane 10 mg l^{-1}, 50 mg l^{-1} and 100 mg l^{-1}) (Fig. 3). No color change was observed in wells without lindane (substrate) (Fig. 3). Quantification of chloride ion concentration showed its gradual increase from 0 day to 15th day of incubation (Fig. 2b) in experimental setup and no significant increase ($p > 0.05$) was observed in control. Increase in chloride ion concentration in inoculated samples was statistically significant ($p < 0.05$) as compared to 0 day. These results depict that *Arthrobacter* sp. T16, isolated from the root surface of *Typha latifolia* have potential to utilize lindane as source of carbon by degrading it. Similar results were obtained during lindane degradation study by *Rhodotorula* sp. VITJzN03 (Salam *et al*., 2013) and *Streptomyces* consortium in slurry system (Saez *et al*., 2015). Also, quite a few reports confirm dehalogenase activity in actinobacteria with lindane as substrate (Cuozzo *et al*., 2009; Fuentes *et al*., 2010a, b; Philip *et al*., 2014).

Lindane degradation kinetics

Lindane biodegradation by *Arthrobacter* sp. T16 was also observed in MSM supplemented with different concentrations of lindane. Maximum degradation was observed at 10 mg l^{-1} lindane followed by 50 mg l^{-1} and 100 mg l^{-1} lindane (Fig. 4), indicating that increasing lindane concentration affected the degradation performance of bacteria. Paul *et al.,* (2013) also observed higher lindane degradation at 10 mg l^{-1} as compared to 100 mg l^{-1} by *Azotobacter chroococcum* strains JL 15 and JL 104. This could be due to inhibitory effect of lindane on growth of bacteria. In our study, average rate of lindane degradation increased with increase in lindane concentration (Table 2) since the rate of reaction, at any time, depends on the concentrations of the reactants at that particular time. Similar results were obtained in studies of lindane biodegradation by Giri *et al*. (2014) and Pesce and Wunderlin (2004). Degradation kinetics of lindane is described well by first order reaction as it was dependent on the substrate concentration (Fig. 5). The kinetic rate constant (k), half life ($t_{1/2}$) and regression coefficient (R^2) for all concentrations of lindane are presented in Table 2.

Protein profile of Arthrobacter sp. T16

To identify lindane induced proteins in *Arthrobacter* sp. T16, comparative analysis of protein profile was done by culturing the bacteria in the presence of glucose as carbon source (without lindane) as well as lindane as the only carbon source. Distinctive polypeptides came into view in lane resultant to MSM supplemented with lindane and these bands were not observed in the culture without lindane (Fig. 6). Bacterial exposure to lindane resulted in the regulative expression of certain proteins. Approximate molecular weight of distinctive polypeptide bands was estimated by Molecular Weight Analysis Tool of Image Lab^{TM} software (version 3.0) using BlueRay prestained protein ladder (10-180kD, GeneDireX^{TM}) as standard molecular weight marker. The molecular weight of polypeptides for *Arthrobacter* sp. T16 was estimated to be 36.6 kD, 43.8 kD and 137.4 kD, for band 1, 2 and 3 respectively.

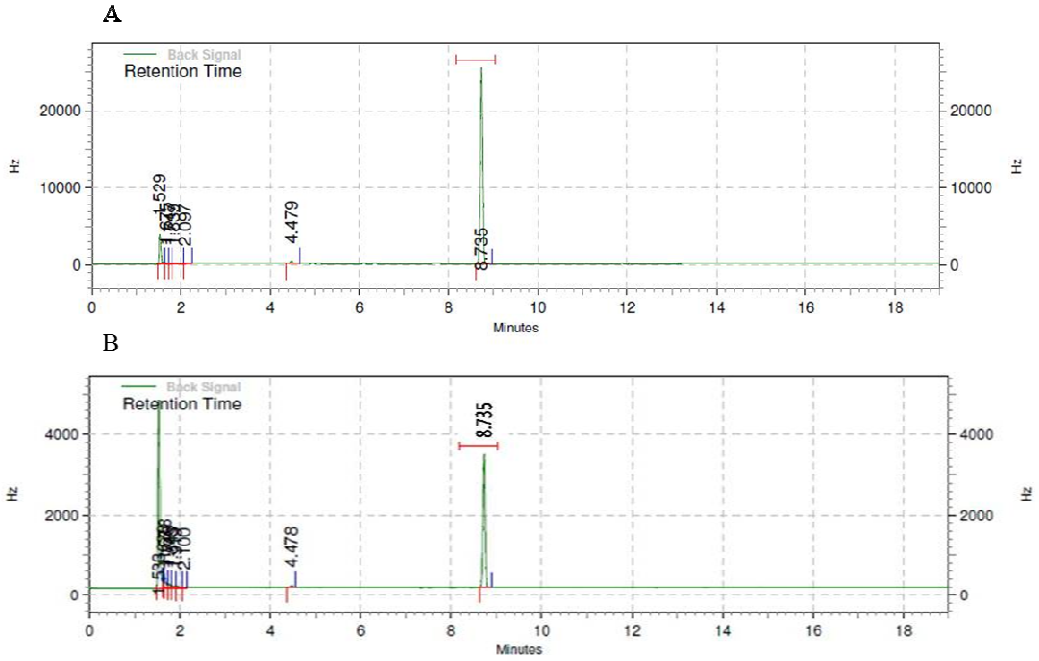

Fig. 1. GC analysis of lindane degradation at 0 day (a) and 15th day (b) by *Arthrobacter* sp. T16. Lindane peak was observed at R_t 8.375 min. Decrease in peak area of lindane shows its degradation

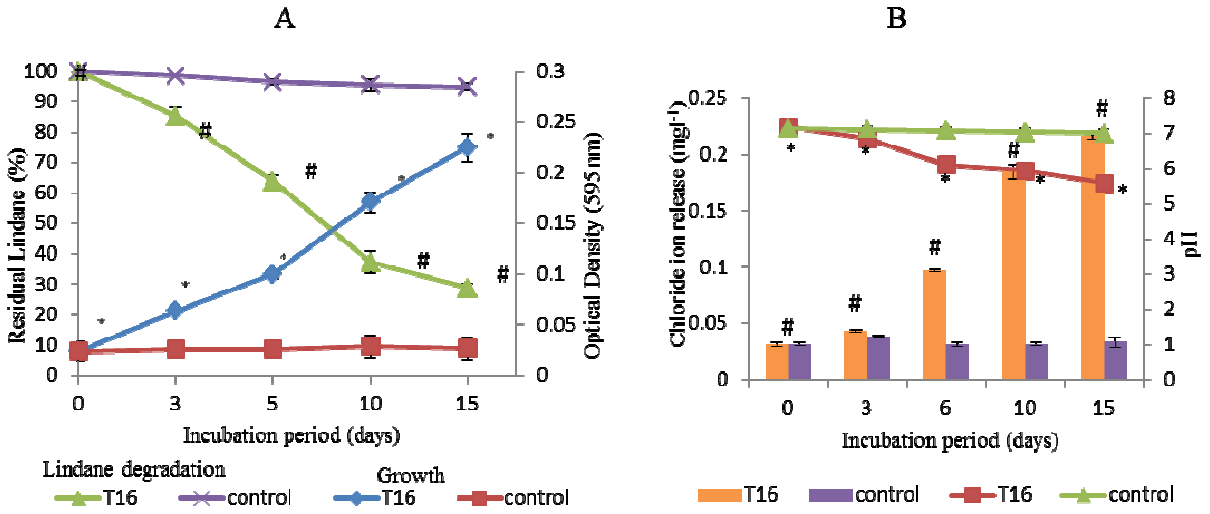

Fig. 2. (A) Degradation of lindane (50 mg l^{-1}) and growth of *Arthrobacter* sp. T16 in liquid culture medium after 15 days of incubation. * Significant difference ($p < 0.05$) in growth of bacteria, # Significant difference ($p < 0.05$) in percentage degradation of lindane by bacterial strain during incubation period of 15 days (B) Chloride ion release and change in pH of broth medium during Lindane degradation by *Arthrobacter* sp. T16.* Significant decrease ($p < 0.05$) in pH, # significant increase ($p < 0.05$) in chloride ion concentration of the culture medium supplemented with lindane from 0 to 15th day. Values are mean of three replicates. Error bars show ± SE

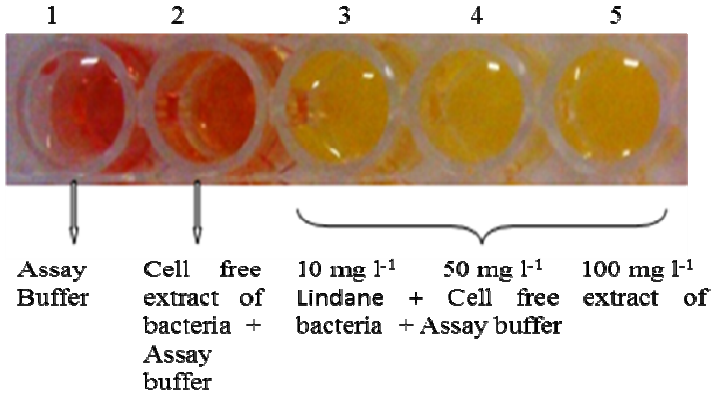

Fig. 3. Lindane dechlorination assay. Well A: assay buffer; B: assay buffer and cell free extract of Arthrobacter sp. T16; C, D, E: assay buffer, cell free extract of bacteria and substrate (lindane 10 mg l^{-1}, 50 mg l^{-1} and 100 mg l^{-1})

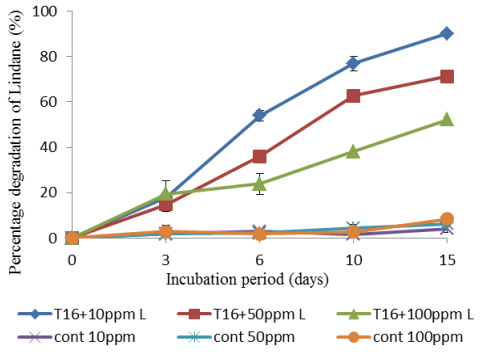

Fig. 4 Biodegradation of lindane at its different concentration (10 mg l^{-1}, 50 mg l^{-1} and 100 mg l^{-1}) by *Arthrobacter* sp. T16. Values are mean of three replicates. Error bars show ± SE. Cont- control

Table 2. Average rate and kinetic parameters for lindane degradation by *Arthrobacter* sp. T16

Lindane concentration (mg l^{-1})	Average rate of degradation (mg l^{-1} day^{-1})	K(d^{-1})	$T_{1/2}$(d)	R^2
10	0.60	0.156	4.44	0.975
50	2.37	0.088	7.9	0.965
100	3.48	0.052	13.3	0.985

Table 3. Significant protein score, sequence coverage, matched protein and mass for unique protein bands of *Arthrobacter* sp. T16

	Significant protein score	Sequence coverage	Matched protein	Mass (kD)
Band 1	283	38%	putative ABC transporter periplasmic amino acid-binding protein	36.8
Band 2	137	50%	elongation factor Tu	43.0
Band 3	57	8%	trifunctional transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	137.3

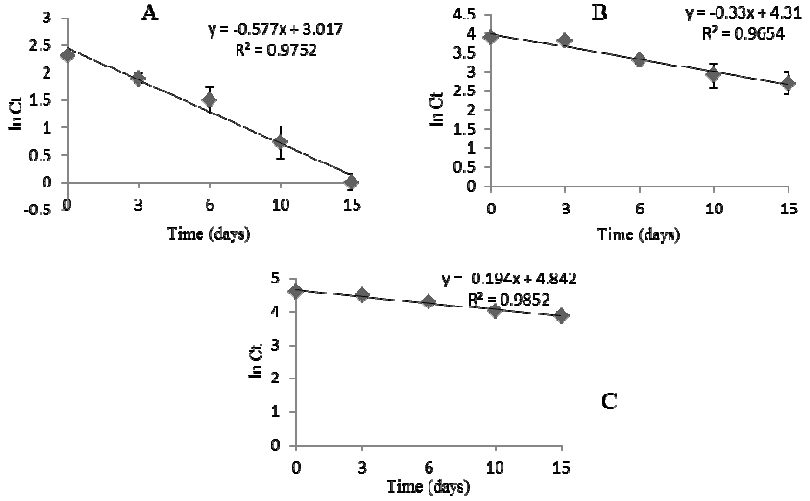

Fig. 5. Kinetic plot of first order reaction for lindane degradation by *Arthrobacter* sp. T16A-10 mg l^{-1} lindane; B-50 mg l^{-1} lindane; C-100 mg l^{-1} lindane

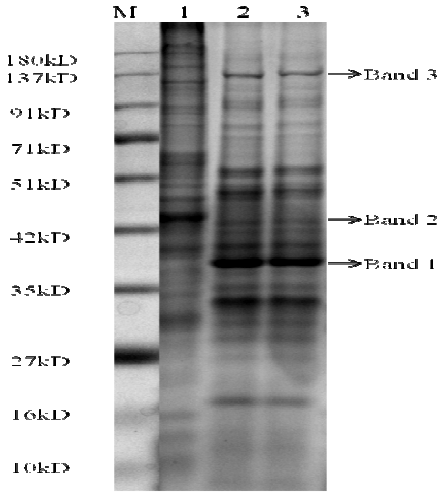

Fig. 6 SDS-PAGE gel presenting protein profile of *Arthrobacter* sp. T16. M: protein marker (10-180 kD). Lane 1: MSM with glucose, lane 2 and 3: MSM with lindane. Band 1,2,3 designate distinctive polypeptide bands in lane 2 and 3

Identification was done by digesting the proteins with trypsin and subjecting to MALDI-TOF and MS/MS analysis. Mass spectra of polypeptides revealed intense peaks which were subjected to Mascot Search against the NCBI database. Table 3 shows the matched protein for each corresponding protein band of *Arthrobacter* sp. T16 with significant protein score and sequence coverage. Polypeptide band 1 was identified as putative ABC transporter periplasmic amino acid-binding protein. This protein is involved in transporter activity which allows the movement of macromolecules, ions and small molecules into or out of the cell. Gram negative bacteria have solute binding proteins dissolved in the periplasm and take part in transmembrane transport. For example, In *Sphingomonas paucimobilis* UT26, genes for ABC-transporter system are necessary for lindane utilization (Endo *et al*., 2007). As a result the extracellular solute-binding protein expressed in the presence of lindane could be involved in lindane transport inside the bacterial cell. Polypeptide band 2 was identified as elongation factor Tu. Elongation factor Tu is one of the prokaryotic elongation factors. Elongation factors are component of the system that synthesizes new proteins by translation at the ribosome. Elongation factor Tu catalyzes the formation of acyl bond between incoming amino acid residue and peptide chain. Elongation factor-Tu may be concerned in protection from stress (Caldas *et al*., 1998) and in our study, this was expressed when bacterial cell was exposed to lindane stress. In *Delftia acidovorans,* the response to chlorophenoxy acid stress showed upregulation of one isoform of elongation factor-Tu (Benndorf *et al*., 2004). Elongation factor-Tu has been reported to be upregulated in *Pseudomonas putida* under toluene stress (Segura *et al*., 2005). Polypeptide band 3 was identified as trifunctional transcriptional regulator / proline

dehydrogenase / pyrroline-5-carboxylate dehydrogenase. Proline is a proteinogenic secondary amino acid, which plays essential role in primary metabolism, osmotic adjustment, redox homeostasis, protection against stress and signaling in all organisms (Servet *et al*., 2012). Proline synthesis takes place from glutamate by delta-1-pyrroline-5-carboxylate synthetase and delta-1-pyrroline-5-carboxylate reductase. Proline gets converted back to glutamate by proline dehydrogenase and delta-1-pyrroline-5-carboxylate dehydrogenase. In gram-negative bacteria, proline dehydrogenase and delta1-pyrroline-5-carboxylate dehydrogenase domains are fused together in single protein known as proline utilization (PutA) protein. In response to environmental stress proline accumulation occurs in bacteria (Liang *et al*., 2013). When *Arthrobacter* sp. T16 was exposed to lindane stress, this protein was expressed. Moreover, Perez-Arellano *et al*. (2010) observed that under stress conditions, some bacteria accumulate proline at a concentration well above which is needed. Therefore, catabolism of proline to glutamate occurs by proline dehydrogenase and delta-1-pyrroline-5-carboxylate dehydrogenase. In another study it has been observed that proline serves as nutrient for *Bacillus subtilis*. These cells seek proline through chemotaxis and then import it through the OpuE-related PutP transporter for catabolism to glutamate (Moses *et al*., 2012; Zaprasis *et al*., 2013).

Therefore, alteration in protein profile and configuration of new proteins might be serving bacteria to bear unfavorable stress environment. Our outcome tend to concur with studies of Rajendran *et al*. (2007) and Kumar *et al*. (2011) that have reported the production of new proteins under stress environment.

Conclusions

This study reveals the presence of lindane degrading bacteria in the roots of wetland plant *Typha latifolia*. To the best of our knowledge, there have been no reports on lindane degrading root epiphytic bacteria from *Typha latifolia*. This study specifies the potential of phytoremediation in controlling the environmental contamination problem with the help of contaminant degrading indigenous organisms present in the roots of plants.

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