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ISOLATION AND ANALYSIS OF BIODEGRADATION POTENTIAL OF THE BACTERIAL ENDOPHYTES *ACINETOBACTER BEIJRENCKII*, B5 AND *ENTEROBACTER AEROGENS*, B8, AGAINST CHLORPYRIFOS *IN VITRO* AND *IN PLANTAE*

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Abstract: Chlorpyrifos is one of the most commonly used organophosphate insecticides that are implicated in serious environmental and human health problems. Herein, we aim to isolate potential bacterial strains that could metabolize chlorpyrifos. For this, we screened the endophytes from the roots of the strawberry plants that could metabolize and degrade chlorpyrifos (10 mg/L). Two strains named B5 and B8 identified that grow at the expense of chlorpyrifos as the sole carbon source. B5 and B8 are able to degrade 100% and 90% chlorpyrifos (10 mg/L) *in vitro* within 72 h and 96 h respectively as tested spectrophotometrically and via Gas Chromatographic (GC) analysis. Furthermore, using pea-seedling as model, our *in plantae* experiments show that chlorpyrifos taken up by the seeds can be efficiently degraded by strains B5 and B8 as reckoned by Gas Chromatographic (GC) analysis. Both the strains, B5 and B8 are able to degrade 97% and 88% of chlorpyrifos (10 mg/L) within 24 h of the treatment. *16S rRNA* gene sequencing revealed that strain B5 showed 99% identity with *Acinetobacter beijrenckii* strain MP17_2B and strain B8 also shared 99% identity with the *Enterobacter aerogens* strain BPRIST043. Additionally the strains also possess biofertilzer activities like phytate production, ammonia production and nitrogen fixation. The results strongly suggest that these strains possess useful bioremediation activities which can be used to improve the quality of food products.

Keywords: Organophosphorous Insecticides; Chlorpyrifos; Endophytes; Biodegradation; Biofertilizer.

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INTRODUCTION

Chlorpyrifos, [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate], has gained a lot of acceptance in agriculture and is an extensively used Organo-phosphorous (OP) insecticide worldwide. It has been widely used to control foliage and soil-borne insect pests. But reportedly, because of its broad spectrum nature chlorpyrifos exposure has shown to have adverse effects on the non-target organisms like aquatic organisms, arthropods including beetles, parasitic wasps, bees and other animals like monkeys, pigs, birds and humans (Cox, 1995). Chlorpyrifos is not toxic in itself but on being transformed by the environment or in vivo by cytochrome 450 it gets converted into a lethal chlorpyrifos-oxon which is about 3000 times more fatal than chlorpyrifos itself (Williamson *et al.,* 2006). Human birth defects and male infertility have also been associated with the exposure to chlorpyrifos. However, its usage has been

restricted in the European countries and the United States but developing countries like India continue to use it to control crop damage from insects and pests in agriculture (Eaton et al., 2008). Not only animals, but in case of plants also it has shown deteriorating trends like fruit deformities (Beck *et al.,* 1991), delayed seedling emergence (Sinclair *et al.,* 1992), abnormal cell division (Amer and Farah, 1983). In this study, we have examined in detail and reported the capabilities of the endophytic bacterial strains, B5 and B8, which can metabolize chlorpyrifos not only *in vitro* but also *in plantae.* The strains were also checked for plant growth promoting attributes like ammonia production, nitrogen fixation and phytate production and their ability to promote plant growth and health using pea seeds as the model system.

EXPERIMENTAL

Isolation of chlorpyrifos resistant endophytic bacteria from Strawberry roots: The roots of the strawberry plants were collected from Solan district of Himachal Pradesh. The endophytic bacteria were isolated from the roots by the method described earlier (Chandrasekhara *et al.,* 2007). The loosely adhered soil was removed, roots were shaken gently and washed carefully under running tap water and then soaked in 70% ethanol for 1 m followed by treatment with 1% sodium hypochlorite and 95% ethanol for 3 m and 30 s respectively. Roots were further rinsed in autoclaved distilled water three times for ten m each and were dried at room temperature. To check the success of surface sterilization, the last wash of sterile water was kept and plated on Nutrient Agar Media plate and incubated at 30˚C for 48 h. No colony formation indicated proper surface sterilization of the roots. Surface sterilized roots were weighed and macerated in pestle and mortar by adding phosphate buffer saline (PBS) (1 mL/g of root) and centrifuged at 1000 rpm for 1 min to remove the debris. Supernatant was collected and centrifuged at 5000 rpm for 10 m. Supernatant was discarded and pellet was re-suspended in 1 mL PBS and serial dilution was placed on nutrient agar media plates and incubated at 30˚C for 72 h to enumerate the colony forming units (CFUs). Colonies that appeared were considered as endophytes and were streaked on nutrient agar plates to obtain pure colonies. The endophytic strains were preserved in 30% glycerol and stored at –80˚C. All the endophytic strains obtained were patched on M9 media supplemented with varying concentrations of chlorpyrifos ranging from 10 mg/L and 2000 mg/L. Strains that were able to utilize chlorpyrifos as the sole carbon source were considered as chlorpyrifos metabolizing isolates and were further analyzed for biodegradation and biofertilzer activities.

Growth kinetics of chlorpyrifos resistant isolates: The bacterial population in the presence and absence of Chlorpyrifos (10 mg/L) was determined by plating the serial dilution and counting the CFUs developed on the nutrient agar plates (Rokade and Mali, 2013). Overnight grown cells were inoculated in 50 mL of M9 media supplemented with chlorpyrifos or glucose (10 mg/mL) to an initial OD_{600} = 1. At different time intervals (0, 24, 48, 72, 96 and 120 h) 500 µL culture was taken out and plated on nutrient agar plates and after incubation at 30 ˚C, CFU/mL was enumerated.

Biodegradation of chlorpyrifos in liquid medium: Bacterial strains were analyzed for their ability to degrade chlorpyrifos in liquid media as described earlier (Kumar S, 2011). Cells were pre-cultured overnight in Nutrient Broth medium and then harvested by centrifugation at 5000 rpm for 7 m, and washed three times with sterile water. After washing, cells (10^7 cells/mL) were inoculated in M9 medium supplemented with chlorpyrifos (10 mg/L). The culture was incubated at 30 ˚C on an incubator shaker at 150 rpm. At regular intervals (24, 48, 72 and 96 h) 5 mL culture was taken and centrifuged at 4000 rpm for 20 m. Then 2.5 mL of supernatant was transferred in another tube and, an equal volume of solvent dichloromethane was added, shaken gently and allowed to settle for 30 m. Then, 2 mL of bottom layer containing chlorpyrifos was analyzed either by UV-Visible spectrophotometry at 260 nm and by Gas chromatography (GC) (Shimadzu GC-2010

Plus). Uninoculated M9 medium supplemented with chlorpyrifos (10 mg/L) was set as control. For GC, the 1µL of the sample was injected at an injection temperature of 280˚C, carrier gas used was hydrogen (3.0 mL/min) and zero air (145 mL/min), split injection mode was set and the column used was RTX-5 with FTD detector and the temperature of the detector was set at 300˚C. Percent degradation was determined by:

$$\text{Percent degradation} = \frac{Pb - Pa}{Pb}$$

Where, Pb: Peak area obtained before degradation
Pa: Peak area obtained after degradation

Endophytic Nature of Isolates: Pea seeds were surface sterilized with 70% ethanol and 1% sodium hypochlorite as described above, placed in petri-dishes containing muslin cloth moistened with distilled water and allowed to grow in the dark for six d at 25°C. After six d, germinated seeds were dampened in a suspension of the bacterial strain (7 log CFU/mL) and were used to check the endophytic nature ofB5 and B8. With this purpose, root and shoot portions were cut at different time points and surface sterilized. After weighing, the samples were minced in 1 mL Mg sol buffer and serial dilutions were placed on LB agar plates to count the CFU. Obtained colonies were matched with the original strain both biochemically and by *16S rRNA* gene sequencing.

Biodegradation of chlorpyrifos *in plantae:* The potential of the endophytic strains to degrade the chlorpyrifos *in plantae* was determined. For this firstly, we evaluated the amount of chlorpyrifos taken up by pea-seedlings (Lee *et al.,* 2012). Pea seeds were surface sterilized with 70% ethanol and 1% sodium hypochlorite and placed in petri-dishes containing muslin cloth moistened with distilled water and allowed to grow in the dark for six d at 25°C. The germinated seeds were then immersed in M9 broth supplemented with 10 mg/L of chlorpyrifos. At regular intervals of 0, 24 and 48 h, 20 seedlings were analyzed by extraction of residual chlorpyrifos absorbed within the seedlings via GC analysis described separately below. Secondly, we evaluated whether the bacterial strains were able to degrade chlorpyrifos taken up by the seedlings. The germinated pea seeds were exposed to M9 broth supplemented with 10 mg/L of chlorpyrifos for 16 h overnight and then dampened in a suspension of bacterial strain (7 log CFU/mL) for 20 m and then incubated at 30°C. Separately, as described below, at regular intervals seedlings were crushed and residual chlorpyrifos was extracted and analyzed by GC. The seedlings were monitored every day for their root length, shoot length, lateral roots, dry weight and wet weight.

Extraction of Chlorpyrifos from the seedlings: The seedlings were subjected to extraction of chlorpyrifos as described earlier (Shang *et al.,* 2001). All the samples were washed with sterile water to remove the traces of remaining chlorpyrifos on the surface. Then they were crushed in liquid nitrogen and transferred to chilled 25 mL tubes. Crushed mass was mixed with 2 mL 10% NaCl solution and the mixed vigorously for 1 min. After that 5 mL of dichloromethane was added, the aqueous extract was clarified by centrifugation at 1000 rpm for 30 m (repeated thrice). The 3.5 mL dichloromethane layer was transferred to 15 mL centrifuge tubes containing 2 g Na_2SO_4 and incubated at room temperature for 1 h. 1 mL dichloromethane layer was removed and run on GC (Shimadzu GC- 2010 Plus). The column used for GC was RTX-5, the injection temperature was 280˚C, the injection mode was split mode, FTD detector was used and the temperature of the detector was set as 300°C.

Analysis of plant growth promoting attributes: Ability of the cells to produce phytase enzyme was analyzed as described earlier (Devi *et al.,* 2015) Cells were grown in liquid media (nutrient broth). 2.5 µL cells at 0.5 OD_{600} were spotted on phytate media plates (sodium phytate or calcium phytate) and kept at 30°C. The bacterial growth was observed for solubilization haloes around the colonies. The zone of p-sol was measured by subtracting the colony diameter from the total diameter after 2d. We also analyzed the nitrogen fixing ability of the strains by its growth in nitrogen-free semisolid medium termed as BAz medium as described earlier (Devi *et al.,* 2015). BAz medium contains azelaic acid as a carbon

source and other nutrients required for bacterial growth, like; K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$, $Na_2MoO_4.H_2O$, $FeCl_3$, bromothymol blue and cycloheximide. BAz medium lacks any nitrogen source. Cells were spotted on BAz agar media and kept for 6 d to observe bacterial growth. *Escehrechia coli* DH5 α was taken as negative control. Ammonia producing ability of the strains was also analyzed as described earlier (Devi *et al.,* 2015). For this, cells were inoculated in 10 mL peptone water to get a final OD_{600} 0.1 incubated for 48 h at 30°C. After 48 h, 0.5 mL Nessler's reagent was added in each tube and change in color was examined. Development of brown to yellow color indicated a positive test for NH_3 production.

16 S *rRNA* gene sequencing: To identify bacterial species, *16S rRNA* gene was amplified using primers FP (5'AGAGTTTGATCCTGGCTCAG) and RP (5'GGTTACCTTGTTACGACTT). The amplified DNA product was sequenced and the *16S rRNA* gene sequence analyzed using the BLAST program.

RESULTS AND DISCUSSION

Isolation and Identification of Chlorpyrifos Resistant Strains: We obtained $5.6X10^5$ CFU/g of strawberry roots. No growth was observed in the last wash suggesting that the cells are endophytes. Thereafter, screening of randomly chosen 100 colonies isolated from the roots of the strawberry plants yielded 19 chlorpyrifos metabolizing strains which were able to grow on the M9 medium supplemented with chlorpyrifos as sole carbon source. These strains were further compared for their strengths to grow on medium containing different concentrations of chlorpyrifos (10 mg/L, 100 mg/L, 500 mg/L,1000 mg/L and 2000 mg/L). Four strains were found to be resistant to 2 g/L concentration out of which two strains (B5 and B8) were able to grow better. For the further studies we choose B5 and B8 owing to their better growth. PCR amplification of the *16S rRNA* gene of B5 and B8 yielded a ~1500 bp amplicons which were sequenced. Comparing the *16S rRNA* gene sequence using BLAST program, we found that B5 is 99% similar to *Acinetobacter beijrenckii* strain MP17_2B (Accession No. JN644620.1) and B8 is 99% similar to *Enterobacter aerogens* BPRIST 043 (Accession No. JF700493.1). Both the DNA sequences were submitted at NCBI with their Accession numbers as KU308266 and KU308267.

Growth Kinetics of Chlorpyrifos Resistant Isolates: Before analyzing the biodegradation capabilities of both the strains, we then tested their growth in the presence of chlorpyrifos. The growth kinetics of both the strains revealed that B5 and B8 grew slightly better in the presence of chlorpyrifos than in glucose (Figure 1A and B). These results suggest that similar to glucose, the strains could utilize chlorpyrifos as carbon source.

Biodegradation of Chlorpyrifos in Liquid Medium: The strains B5 and B8 were checked for their ability to degrade chlorpyrifos *in vitro* after extracting the chlorpyrifos at different time intervals using the UV-Visible spectrophotometric detection method. As reported previously, the maxima for chlorpyrifos was 260 nm (Jing-Liang *et al.,* 2006). It was observed that the strain B5 as compared to B8, degraded chlorpyrifos better after 24 hrs of incubation (Figure 1C). But this could be due to the slow growth of B8 during 24 h (Figure 1 B). At 96 h, B5 and B8 degraded 100% and 93% of chlorpyrifos. These results were further reconfirmed and substantiated by GC analysis wherein the strains B5 and B8 were able to degrade 100% and 90% of chlorpyrifos within 72 h and 96 h respectively (Figure 2A and 2B).

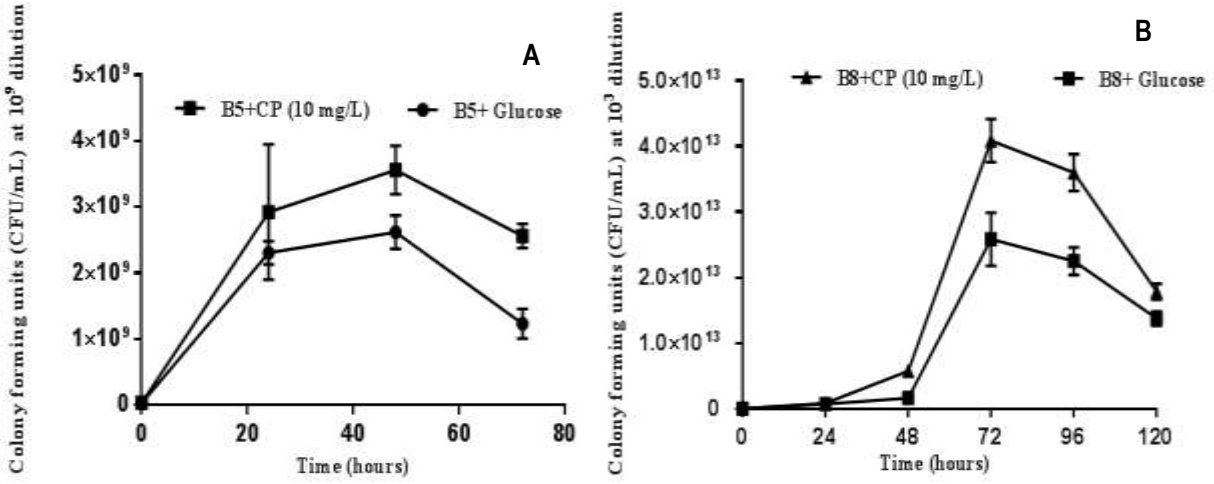
B5 and B8 are Endophytic in Nature: On evaluation of the endophytic nature of B5 and B8 by extracting endophytes from seeds treated with B5 and B8, we found the presence of both B5 and B8 on the second d of treatment in root samples. B8 showed up in the shoots after 24 h and B5 at 48 h. We further observed a gradual increase in the number of B5 and B8 colonies in both the shoots and roots of seedlings (Figure 2C). Morphology of all the colonies obtained after plating the extracted endophytic samples was found to be similar to that of B5 and B8. Moreover, their identity was confirmed by sequencing *16S rRNA* gene amplified from genomic DNA isolated from 10

colonies that were randomly picked. Therefore, we concluded that both the strains, B5 and B8, isolated from roots of the strawberry plants are endophytic in nature.

Chlorpyrifos uptake by the Pea Seedlings: To study whether chlorpyrifos can be taken up by the pea seedlings from the nutrient solution, the pea seedlings were immersed in chlorpyrifos and then the concentration of accumulated chlorpyrifos was determined by the extraction of chlorpyrifos from the pea seedlings at regular intervals of time. The peak of chlorpyrifos was obtained at 7.59 retention time. At 0 h the peak area as observed in the GC chromatogram was 11,955.1 which further increased to 1,19,419.5 and 45,99,313.0 in 24 and 48 h, respectively. The gradual increase in the peak area was an indication that with passage of time chlorpyrifos was accumulating inside the pea seedlings (Figure 3).

Biodegradation of Chlorpyrifos *in plantae:* We also tested if the accumulated chlorpyrifos can be degraded by the endophytes which can enter into the seedlings efficiently at 48 h (Figure 3C). As compared to the uninoculated seedlings (peak area: 1,55,766), B8 treated seedlings contain only 12 % chlorpyrifos (peak area:17,901) suggesting a degradation of 88 % in 24 h. Interestingly at 48 h no peak was observed. Surprisingly, we found that after 24 h in the B5 treated seeds, the seedlings contain only 3% chlorpyrifos (peak area 4,182) as compared to the untreated seeds (Figure 3B and 3D). Similar to B8, B5 degraded the chlorpyrifos completely in 48 h. both the strains were able to enter the seedlings at 24 h, it could be possible that the resident endophytes were able to degrade the chlorpyrifos. Looking at the treated seedlings, we found that after 12 d, the chlorpyrifos treated seedlings showed a significant reduced root and shoot length as compared to untreated seedlings. Remarkably, the bacterized seeds either with B5 or B8, rescued the growth defects in seedlings caused by chlorpyrifos.

Analysis of Plant Growth Promoting Activities: The strains B5 and B8 were found to produce 1.45±0.05 cm and 1.08±0.15 cm clear halo zones around the colonies after 48 h of incubation as shown in (Figure 4A), suggesting that they could solubilize calcium phytate and form peripheral halo zone on phytase specific agar medium around the colonies. Nitrogen fixation was reported as an important plant growth promoting trait of root nodulating bacteria. We found that both, B5 and B8, were able to grow on the nitrogen free medium (Figure 4B). Additionally, we found that both the strains B5 and B8 were able to convert the freely available atmospheric nitrogen into ammonia as indicated by the change in the color of the medium on the addition of Nessler's reagent as shown in (Figure 4C). These traits might help these bacteria to establish a symbiotic relationship with the plants.



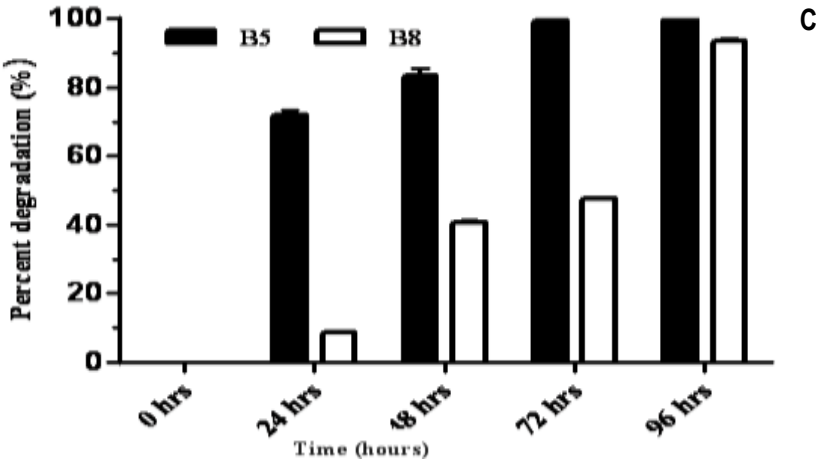


Figure 1 (A) Growth curve of B5 in the presence and absence of chlorpyrifos (10 mg/L). Cells were inoculated in triplicates in modified M9 liquid medium containing glucose (•) and chlorpyrifos (■), as described in experimental procedures and the CFUs was enumerated at different time intervals by plating the serial dilutions. (B) Growth curve of B8 in the presence and absence of chlorpyrifos (10 mg/l). Cells were inoculated in triplicates in modified M9 liquid medium containing glucose (•) and chlorpyrifos (■), as described in experimental procedures and the CFUs was enumerated at different time intervals by plating the serial dilutions.(C) Degradation of chlorpyrifos (10 mg/L) by B8 and B5. Cells were grown in triplicate as described in the experimental proceduresin the presence of 10 mg/l chlorpyrifos in M9 liquid medium. At different time intervals chlorpyrifos was extracted from the culture supernatant using equal volume of dichloromethane. Amount of chlorpyrifos degraded was calculated using spectrophotometer at 260 nm. Percentage degradation was calculated by comparing with control medium lacking B5 and B8 cells.

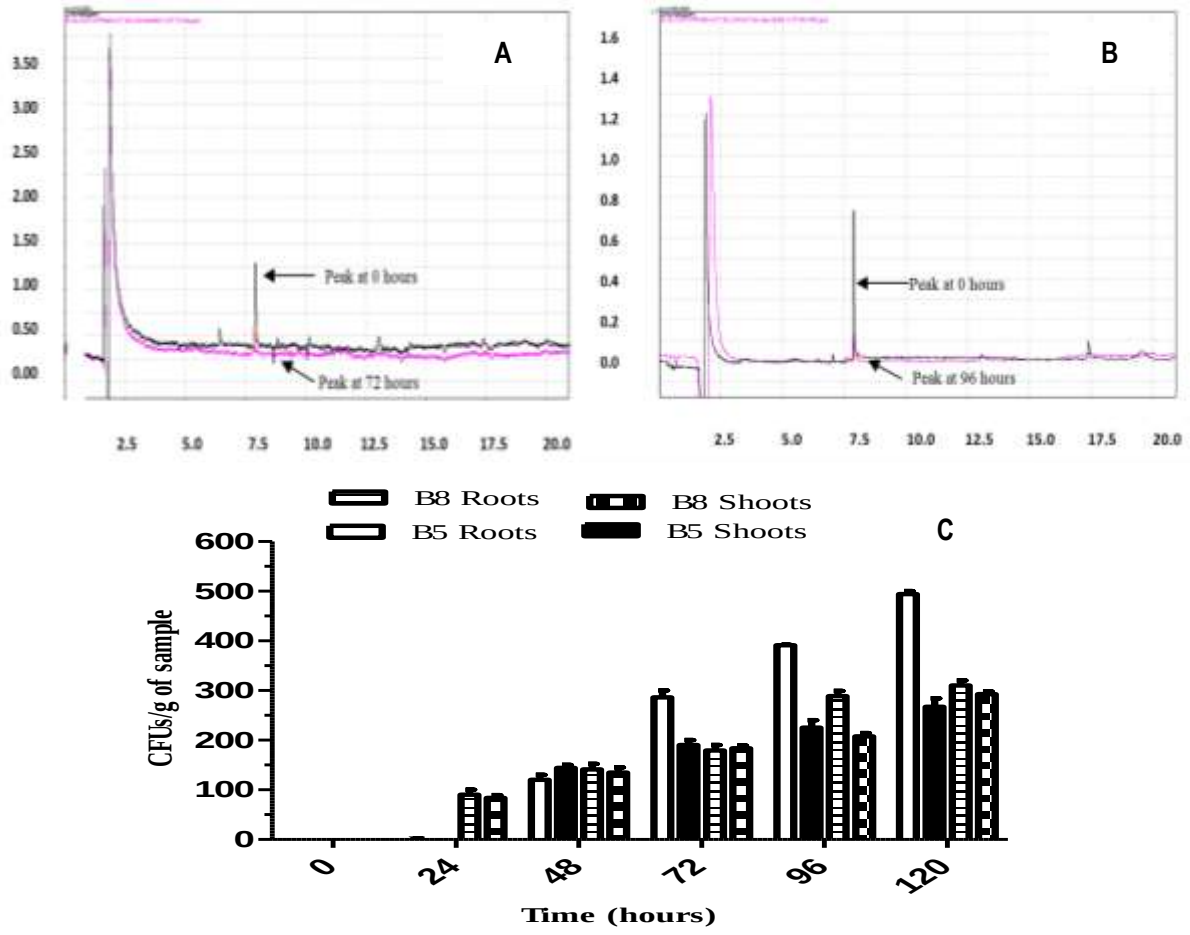


Figure 2. Degradation of chlorpyrifos (10 mg/L) by B8 and B5

(A) Cells were grown in triplicate as described in Figure 2 (A) in the presence of 10 mg/L chlorpyrifos in M9 liquid medium at different time intervals chlorpyrifos was extracted from the culture supernatant using equal volume of dichloromethane. Amount of chlorpyrifos was calculated using Gas chromatographic analysis. Percentage degradation was calculated by comparing with control medium lacking B5 cells. **(B)** Cells were grown in triplicate as described above in (A) in the presence of 10 mg/L chlorpyrifos in M9 liquid medium. At different time intervals chlorpyrifos was extracted from the culture supernatant using equal volume of dichloromethane. Amount of chlorpyrifos was calculated using Gas chromatographic analysis. Percentage degradation was calculated by comparing with control medium lacking B8 cells. **(C) B5 and B8 are endophytic.** Seeds were treated as described in the experimental procedures and root and shoot samples were excised at mentioned time points and surface sterilized. The samples were minced in Mgsol and CFUs were enumerated after plating serial dilutions on LB agar media.

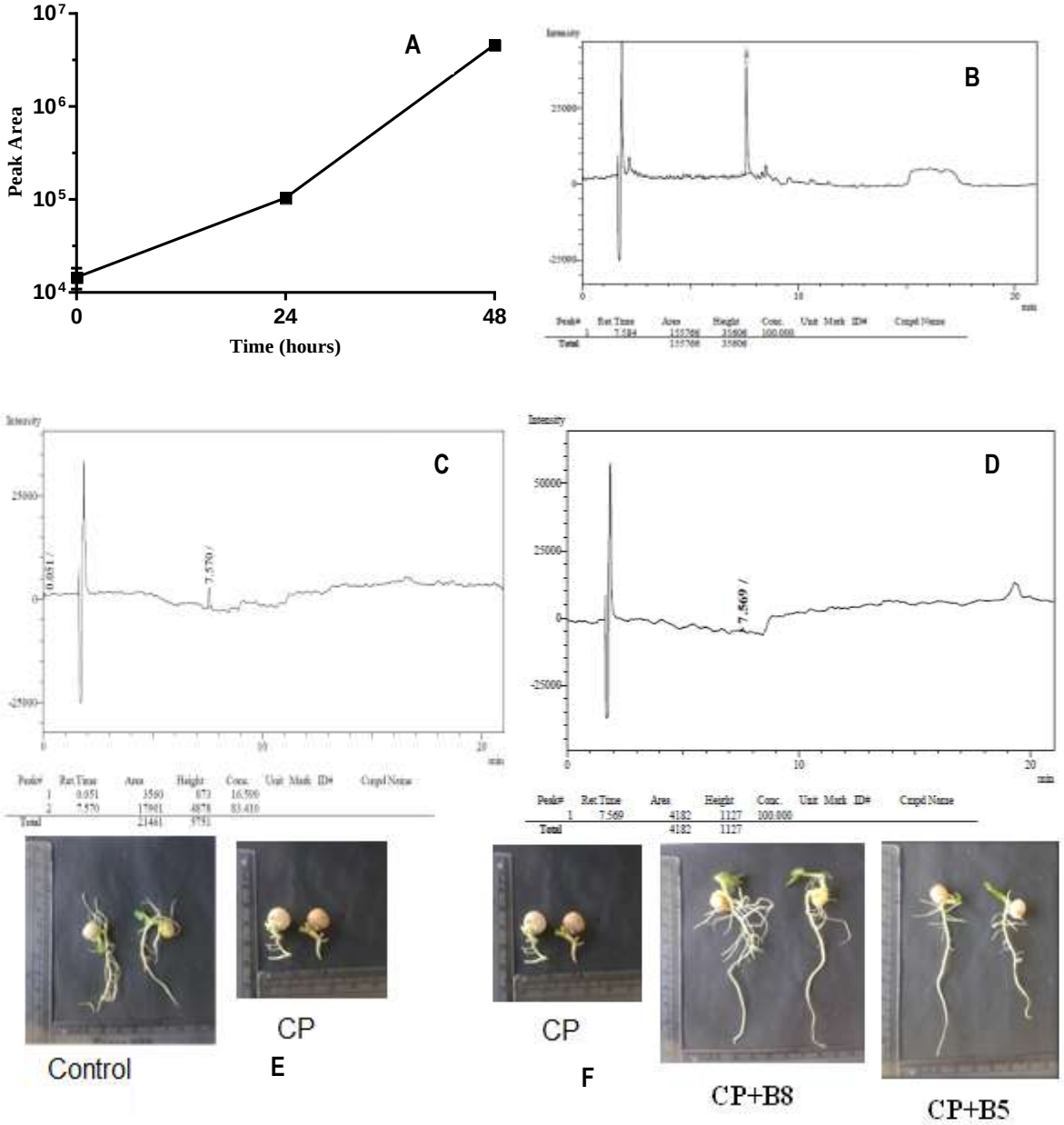


Figure 3. Chlorpyrifos uptake by Pea Seedlings (A) Accumulation of chlorpyrifos by the pea seeds from the nutrient solution supplemented with chlorpyrifos 10 mg/L and determination of residual chlorpyrifos by extraction at regular intervals via GC analysis. **(B)** GC chromatogram of accumulated chlorpyrifos (standard) **(C)** GC chromatogram depicting degradation of accumulated chlorpyrifos by the bacterial strain B8 after 24 hours of treatment. **(D)** GC chromatogram depicting degradation of accumulated chlorpyrifos by the bacterial strain B5 after 24 hours of treatment. **(E)** Growth of the inoculated pea seeds and pea seeds treated with chlorpyrifos. **(F)** B5 and B8 stimulates root and shoot formation and inhibit the adverse effects caused by chlorpyrifos.

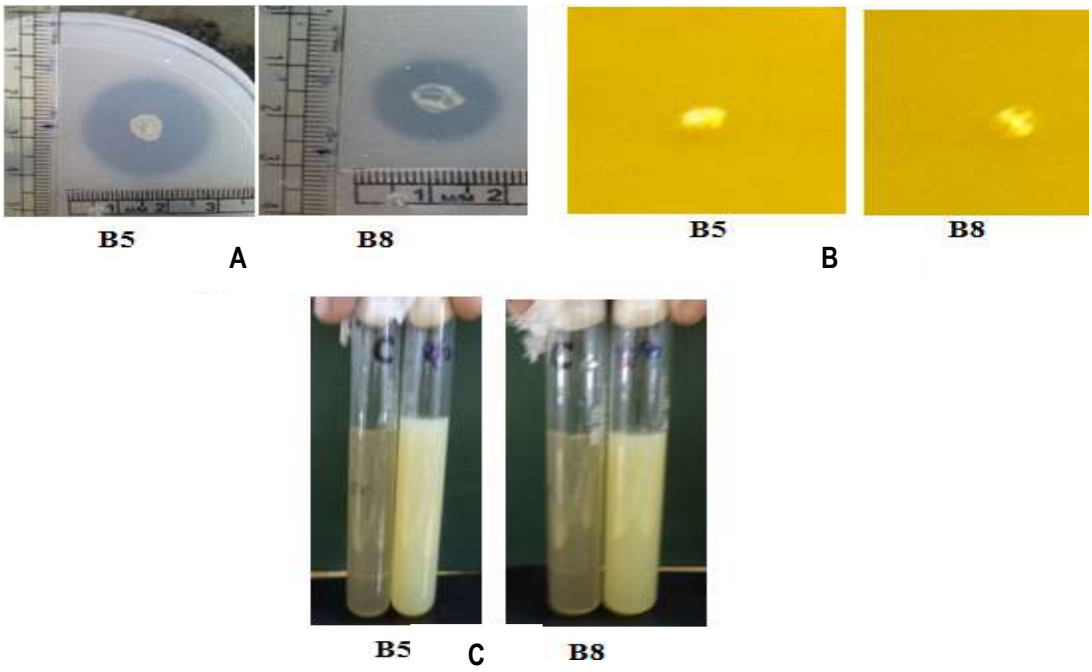


Figure 4. Biofertilizer activities of B5 and B8 strains. (A) Phytase activity. Plates having phytase specific agar medium carrying calcium phytate were spotted with strains B5 and B8 and analyzed for 48 h for the formation of halo. **(B) Nitrogen fixation**. Plates having Basal Media without any nitrogen source were spotted with B5 and B8 strains and analyzed for 24 hours for their growth**. (C) Ammonia production.** The accumulation of ammonia was detected by adding Nessler's reagent. A deep yellow to brownish color production indicated production of ammonia by the endophytic bacterial strain B5 and B8. There was no color change in the negative control.

It has been reported earlier that chlorpyrifos is toxic to plants and thus could reduce the productivity. Chlorpyrifos has been shown to be toxic to a variety of plants like alfalfa (*Medicago sativa*), clover (*Melilotu alba* and *Trifolium pratense*) (Smith *et al.* 1978), *Pinus halepensis* and *Arabidopsis thaliana* (Olofinboba and Kozlowski, 1982 and Aben *et al*., 1992). Besides plants, its exposure kills beneficial arthropods including bees, ladybird beetles and parasitic wasps, and other animals including fish and other aquatic organisms and humans, because of their broad spectrum effects on non-target organisms (Cox 1995). Chlorpyrifos also affects the availability of vital plant nutrients (P, N and K) in the soil and also decrease the content of P and N content in soil treated with chlorpyrifos. Previously it has been shown that negligible amount of chlorpyrifos enters the plants via the roots (Smith *et al.* 1967) indicating its non-systemic nature but herein, we have found that substantial amount of chlorpyrifos enters the pea plants and it has an inhibitory effect on the root length, shoot length, number of lateral roots and the general health of the plants. Plants might contain endophytes to combat these stresses. And with this hypothesis we isolated a bacterium that not only metabolizes chlorpyrifos but also have the potential to enhance the nutritional status of the plant by making the nutrients like phosphorous and nitrogen available to the plants. Using GC and *in plantae* experiments we showed that the chlorpyrifos degrading bacteria significantly reduces the accumulation of chlorpyrifos which leads to improvement in the growth of seedlings. The dramatic decline of the accumulated chlorpyrifos indicates that the isolated strains were efficacious in metabolizing and degrading the chlorpyrifos in the plant tissues. Hence, they are efficient tools that can help in remediating chlorpyrifos polluted sites and also help in enhancing the nutritional status of the plants.

CONCLUSION

With the increasing population and urbanization, expanding the agricultural land is difficult and is limited by a lot of constraints such as competing with urban growth and scarcity of fresh water. Therefore, improvement of agricultural productivity and reducing the

mass damage caused to the agricultural land and human health by the persistent use of chemical insecticides and chemical fertilizers will be a key approach for reducing the global food security over the coming decades. This report demonstrates that the isolated endophytic bacterial strains can metabolize chlorpyrifos as the sole carbon source for their growth in both solid and liquid medium. The strains also have the potential to degrade chlorpyrifos *in vitro* and *in plantae*. The strains also possess the ability to enhance the nutritional status of the plants by making organic phosphorous and nitrogen available to the plants and thereby improve the general health of the plants. Hence, these strains could be helpful in decreasing the toxicity of pesticides. We are in process to study the degradation potential of these strains on other class of pesticides.

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