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Biodegradation of Chlorpyrifos Using Indigenous *Pseudomonas* sp. Isolated from Industrial Drain

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Abstract: Excessive use of pesticides are disturbing major components of ecosystems. Therefore their removal using biodegradation technique is the need of time. In present study, 35 microbial strains were isolated from industrial drain which carries effluents from chlorpyrifos manufacturing plant. These strains significantly differ in their ability against chlorpyrifos resistance and degradation. Out of these strains WW5 was found most resistant and effective in chlorpyrifos degradation. On the basis of morphological, biochemical and physiological characteristics, strain WW5 was identified as *Pseudomonas* sp. Biodegradation potential of WW5 strain was studied under different culture conditions like concentration of chlorpyrifos, carbon sources, pH and inoculum densities. In the presence of glucose chlorpyrifos was co-metabolized. High pH (8) and high inoculum density (10^8 CFU/mL) show most efficient results in biodegradation. Presence of other nutrients enhanced chlorpyrifos degradation probably due to high growth on easily metabolizable compounds which in turn favors biodegradation. The strain WW5 showed 94% degradation of chlorpyrifos (400 mg/L) within 18 days of incubation. This strain can be used for bioremediation and ecological restoration of sites, contaminated with chlorpyrifos.

Key words: Biodegradation, bioremediation, organochlorines, chlorpyrifos, *Pseudomonas*

INTRODUCTION

Pesticides were developed to meet the world's food demand but with excessive use they are becoming necessary evil. Pesticides are now disturbing major components of ecosystems being persistent in environment (Gavrilescu, 2005). Organochlorines pesticides have been replace by Organophosphorus Pesticides (OP). Organochlorines are toxic for non-target organisms and remain in environment for longer duration (Tariq *et al*., 2007), whereas organophosphates are neurotoxic and suppress acetylcholine esterase (AChE). It regulates nerve impulse transmission by lowering the acetylcholine concentration. OP causes inactivation of AChE, boosts acetylcholine, continuously stimulates nerve fibers and eventually leads to tetany and exhaustion (Singh *et al*., 2009).

Chlorpyrifos [CP; O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate); $C_9H_{11}Cl_3NO_3PS$] is an organo-phosphate pesticides, broadly used on vegetables, cereals and cotton for controlling pests. For domestic purposes it is used against flies, mosquitoes and other house hold pests (Li *et al*., 2010). Harmful effects of chlorpyrifos include twitching of muscles, skin irritation, depression, respiratory failures, convulsion, subtle neurological effects and death (Anwar *et al*., 2009). Chlorpyrifos may persists in environment up to one year (half-life of 60-120 days), have low water solubility (2 mg/L) and soluble in organic solvents. It bioaccumulates in aquatic plants, blue-green algae, mosquito fish and goldfish. Only limited information is available on the fate of chlorpyrifos in the soilBcrop system (Tariq *et al*., 2007). Chlorpyrifos degrades into number of toxic products which have bioaccumulation tendency (Fang *et al*., 2008). 3,5,6-trichloro-2-pyridinol (TCP) is the main metabolite of chlorpyrifos having antimic, robial properties. United States Environmental Protection Agency classified TCP as toxic, antimicrobial, more mobile than chlorpyrifos, greater water solubility, wide spread in environment and persistent having half-life of 65-360 days (Thengodkar and Sivakami, 2010).

Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state or to levels below concentration limits, established by regulatory authorities. Out of number of bioremediation techniques, one that involves microbes has received much attention for the cleaning of contaminated environment (Struthers *et al*., 1998). Microbes that are present in contaminated sites for a longer period of time develop the ability to degrade/tolerate such contaminant. Similarly, microbes which are present in agricultural soils or wastewater streams experience repeated exposures of pesticides. Such microbes with advanced/new traits can be used for pesticide degradation. Several unsuccessful efforts were made, to isolate a chlorpyrifos degrading microbial system (Thengodkar and Sivakami, 2010). One of the

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reason behind such failure is that the chlorpyrifos metabolize/degrade into 3,5,6-trichloro-2-pyridinol (TCP) and has antimicrobial effects. Therefore, interest has been shifted to use indigenous microorganisms for biodegradation and removal of toxicants (Mukherjee *et al.*, 2004).

Earlier studies reported number of microorganisms having variable potential for chlorpyrifos biodegradation like; *Aspergillus* sp. Y, *Trichoderma* (Liu *et al.*, 2003), *Fusarium* (Wang *et al.*, 2005), *Ralstonia* sp. (Li *et al.*, 2010), *Pseudomonas* ATCC700113 (Feng *et al.*, 1997), *Bacillus pumilus* C2A1 (Anwar *et al.*, 2009), *Pseudomonas aeruginosa*, *Bacillus cereus*, *Klebsiella* sp., *Paracoccus* sp. (Xu *et al.*, 2008), *Sphingomonas*, *Stenotrophomonas*, *Bacillus* sp. *Brevundimonas*, *Pseudomonas* sp. (Li *et al.*, 2008), *Pseudomonas* sp. (Singh *et al.*, 2009), *Klebsiella* sp. (Ghanem *et al.*, 2007), *Alcaligenes faecalis* (Yang *et al.*, 2005), *Enterobacter* strain B-14 (Singh *et al.*, 2004), *Spirulina* platensis (Thengodkar and Sivakami, 2010), *Chlorella vulgaris* (Mukherjee *et al.*, 2004), *Verticillium* sp. DSP strain (Fang *et al.*, 2008)

Commercialization of bioremediation has not been established fully, limited experimentation of changes in bacterial communities is one of the reasons. Both biotic (microbial agent, competition, growth kinetics, inoculum density) and abiotic factor (pH, temperature, moisture, nutrients availability) effects the biodegradation process (Xie *et al.*, 2008). Singh *et al.* (2008) reported that high pH and inoculum density (CFU/mL) are critical parameters for chlorpyrifos degradation and for in-situ bioremediation, cell level of 10^6-10^8/g soil is suggested. In contrast, 10^5 cells g/G of *Agroacterium* strain was sufficient for rapid atrazine degradation. Increase in pesticide concentration yaer by year is another source of in-situ bioremediation failure (Struthers *et al.*, 1998).

For this study we isolated bacteria from effluent of pesticide processing factory. These bacteria were then isolated on the basis of chlorpyrifos tolerance and effectiveness in degradation. Major factors (pesticide concentration, carbon sources and pH and inoculum size) that influence chlorpyrifos biodegradation by these newly isolated bacterial cultures were studied.

MATERIALS AND METHODS

Chemicals and soil sampling: Throughout the experiment only analytical grade chemicals were used. Chlorpyrifos (95%) was obtained from Pak China Chemicals, Lahore. Sludge samples were collected using standard protocols of Ghanem *et al.* (2007), from wastewater drain of industrial area carrying effluents from pesticide manufacturing factories.

Enrichment, isolation and selection of microbial strains: Chlorpyrifos resistant microbial strain were isolated from collected samples using Minimal Salt Medium (MSM) at pH 7. The medium contained: 200mg $MgSO_4$, 900mg K_2HPO_4, 200mg KCl, 2mg $FeSO_4$, 2mg $MnSO_4$, 2mg $ZnSO_4$ and 1000mg NH_4NO_3/L. About 25g of soil sample, 25ml/L chlorpyrifos and 200mL sterile MSM were added in 250ml flask and shaked at 100rpm. 1 week later 20ml of the culture was transferred to fresh MSM containing more concentration of pesticide as used previous week. For the maintenance of moisture sterile distilled water was used. After every week 10ml culture was transferred to fresh MSM and pesticide concentration of 150ml/L chlorpyrifos was achieved.

10-fold serial dilutions were prepared from the last sub-culture and 120 µL of each dilution was spread on nutrient agar plate containing 150ml/L chlorpyrifos. Colonies that grow on nutrient agar (containing chlorpyrifos) were isolated and purified using streak plate method. Strain exhibiting the maximum growth potential was selected for further studies (Ortiz-Hernandez and Sanchez-Salinas, 2010).

Taxonomic identification of the microbial strain: Isolated strains were identified using Bergey's Manual of Determinative Bacteriology, on the basis of physiological, morphological and biochemical properties (Holt *et al.*, 1994).

Inoculum preparation: Isolated/selected strain was grown in nutrient broth. For the preparation of seed culture, broth culture was centrifuged for 10min at 4600rpm. It was washed with sterile N-saline (0.9%) then resuspended (in 0.9% N-saline) and OD_{550} of 0.5 was set. This suspension was quantified as colony forming units (CFU/mL) by the technique of dilution plate count (Fang *et al.*, 2008).

Biodegradation of chlorpyrifos: To study the potential of isolated strain for chlorpyrifos biodegradation, shake flask studies were conducted. 25mL MSM (sterile), inoculum (10^4 CFU/mL) and known concentration of chlorpyrifos was mixed in a flask. Flask was shaked (100rpm) and incubated (37°C) for 20 days. In a control flask, 25mL MSM (sterile) and known concentration of pesticide was added. Control was not inoculated with isolated strain. All the experiments were conducted in triplicate. After regular interval (24h) samples from these flasks were drawn (aseptically) and were analyzed for remaining pesticides concentration (Fang *et al.*, 2008). Current study targets the biodegradation of chlorpyrifos only not its metabolites.

Extraction and HPLC analysis of chlorpyrifos: For HPLC analysis the extracted samples were centrifuged (at 7200rpm) for about 10min and supernatant was mixed with equal volume of dichloromethane (DCM). Organic layer was collected and DCM was evaporated (under nitrogen) at room temperature. Residues were

filtered using flouropore™ filter membrane (0.45 µm diameter) after dissolving in acetonitrile (Ortiz-Hernandez and Sanchez-Salinas, 2010). Varian HPLC (equipped with a ternary gradient pump, UV detector, electric sample valve, column oven and C18 reversed-phase column) was used for pesticide analysis using mobile phase of methanol : water (85:15, v:v). HPLC conditions were set as follows, sample volume: 20 µL, flow rate: 1 mL/min, retention time: 15min and wavelength: 290nm (Li *et al*., 2007)

Effect of pesticide concentration: Pesticide concentration of 100-600mg/L was used to study the effect of concentration on biodegradation process. Measured concentration of chlorpyrifos, 150mL MSN (sterile) and inoculum (10^4 CFU/mL) were mixed, incubated (37°C) and shaked continuously at 100rpm. Same setup without inoculum was kept as control. All the setups were established in triplicate. After every 24h, samples (10ml) were withdrawn and pesticide residues were extracted and analyzed (using procedure as described in previous sections).

Effect of carbon sources: Different carbon sources tested for enhancement of chlorpyrifos degradation were glucose, mannose, yeast extract, galactose and starch. Carbon source (5%), MSN (150mL), chlorpyrifos (400mg/L) and inoculum (10^4 CFU/mL) were aseptically added in the flasks (triplicate). Same setup without added carbon source was kept as control. These flasks were incubated (37°C) at 100rpm and pesticide concentration was determined after every 24h.

Effect of inoculum concentration: Inoculum ranges of 10^3-10^8 CFU/mL were prepared by adding appropriate amount of seed culture. This inoculum, MSN (150mL), chlorpyrifos (400 mg/L) was aseptically added in the flasks (triplicate). Same setup without inoculum was kept as control. These flasks were incubated (37°C) at 100rpm and pesticide concentration was determined after every 24h.

Effect of pH: pH range of 6-8.5 was tested in order to optimize chlorpyrifos degradation. Experiments were setup in triplicate at 37°C using 150mL MSM, 400 mg/L chlorpyrifos and 10^4 (CFU/mL) inoculum density. Pesticide concentration was determined after every 24h.

Statistical analysis: Data was analyzed using Costat and SPSS software.

RESULTS AND DISCUSSION

Bioremediation of bound and aged pesticide provides the eco-friendly solution for contaminated environment. For this purpose indigenous bacteria are considered best option as they are most adaptive with the native environment and secondly they do not pose any threat to native flora and fauna (Thengodkar and Sivakami, 2010). Number of condition increases or decreases the success of biodegradation like; pH, temperature, moisture, nutrients, microbial agent, competitive capability, pesticide bioavailability, etc. (Mukherjee *et al*., 2004). Soil decontamination by using isolated microbe has been successful carried out by many researchers. For instance, Dams *et al*. (2007) isolated *Sphingobium chlorophenolicum* from soil and used it against pentachlorophenol (PCP). This strain exhibit 80% degradation compared to control (non-inoculated soils). Similarly *Pseudomonas putida* ZWL73 accelerated the biodegradation of 4-chloronitobenzene (4CNB) in contaminated soil (Niu *et al*., 2009). Hong *et al*. (2007) reported complete decontamination of soil containing fenitrothion (50 mg/kg) by *Burkholderia* sp. in 15 days, whereas control was able to degrade only 30.4%. In Antarctic soil, oil contamination was removed up to 75% by psychrotolerant strain (*Acinetobacter johnsonii)* whereas autochthonous bacterial communities degrade only 35% of oil (Ruberto *et al*., 2003). Studies have also successfully used filamentous fungi for bioremediation. *Rhizopus* sp., *Penicillium funiculosum* and *Aspergillus sydowii* has the potential to degrade petroleum hydrocarbons up to 36% (Mancera-Lo´pez *et al*., 2008). *Absidia cylindrospora*, Another filamentous strain degraded 99% of fluorine within 288h in soil slurry, while in non-inoculated soil the same fluorine degradation lasted for 576h (Garon *et al*., 2004). Biodegradation is a composite method, where each step is catalyzed by specific enzyme. Absence of such enzyme is considered as most common reason for pesticide persistence. Complete biodegradation yield carbon source and energy by the process of oxidation. This carbon and energy is used for the growth of microorganisms. In environment, where pesticide resistant microorganism is not present or if population size has reduced due to pesticide toxicity, in that situation a selected microorganism can be introduced for enhance biodegradation (Niu *et al*., 2009).

Isolation and characterization of chlorpyrifos degrading Bacterium: For this study, chlorpyrifos resistant bacteria were screened by enriching wastewater samples from industrial drain which receives effluent from pesticide manufacturing plant. 35 strains were isolated based on their morphological characteristics. Isolates were allowed to grow on MSM (containing chlorpyrifos up to 150 mg/L). Only WW5 showed very good growth and is used for in-depth study of chlorpyrifos degradation, other isolated were sensitive to the increasing concentration of chlorpyrifos (Fig. 1).

Identification of strain: Colonies of strain WW5 were gram negative, rod shaped, motile, aerobic and show

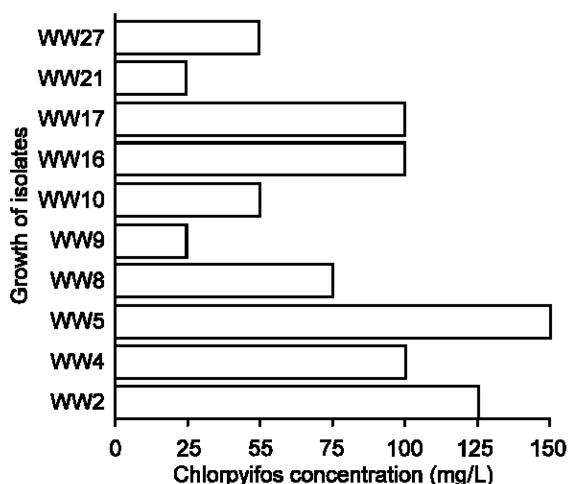

Fig. 1: Screening of isolated on the basis of resistance in chlorpyrifos amended medium

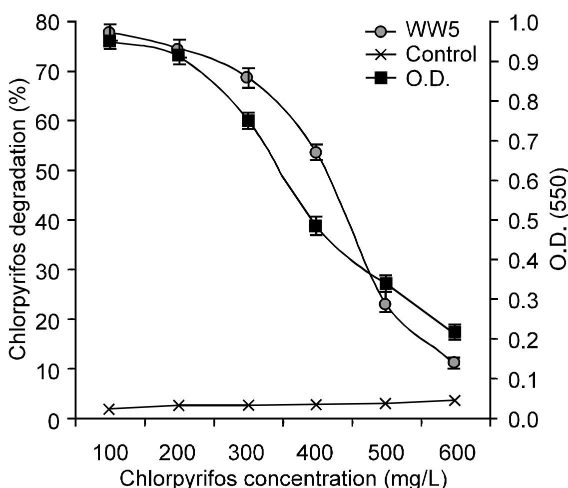

Fig. 2: Effect of concentration on chlorpyrifos degradation. pH: 7, inoculum density: 10^4 (CFU/mL), incubation: 21 days and no added supplement

positive catalase and oxidase test. WW5 was identified as pseudomonas sp. by standard protocol set in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). To our understanding, this is the enhanced biodegradation of chlorpyrifos by *Pseudomonas* sp. Pseudomonas is a versatile genus and previous reports suggested that this genus could degrade a number of chemicals, including pesticides like, carbaryl (Swetha and Phale, 2005), malathion (Imran *et al.*, 2004), propargite (Sarkar *et al.*, 2010) and present study also supported their immense biodegradation diversity.

Effect of chlorpyrifos concentration: Pesticide concentration is one of the major factors that determine the fate of biodegradation. Very high concentration usually leads to the failure of biodegradation as microbes are not resistant against that. On the other hand very low pesticide concentration shows strong affinity with soil particles thus become non-available to microbes. The maximum degradation of 78% was achieved in 18 days of incubation (Fig. 2). *Pseudomonas* is best adaptive at low concentration, the chlorpyrifos degradation decreases with the increase in concentration. At concentration of 100-300mg/L the degradation decrease gradually but from 300mg/L to onward the decline in degradation was sharp. The growth of *Pseudomonas* in term of OD_{550} also degrease with increase in chlorpyrifos concentration. These results are in correspondence with those of Singh *et al.* (2004), who reported maximum biodegradation of chlorpyrifos at 250 mg/L. As the concentrations of pesticide increases longer lag phases were observed. Karpouzas and Walker (2000) suggested that these longer lag phases might because of the requirement of larger microbial number and acclimation period to begin enhanced biodegradation. Conversantly, Struthers *et al.* (1998) reported ethoprophos degradation at high concentrations. To our best knowledge this *Pseudomonas* sp. has enhanced abilities for chlorpyrifos degradation. Boettcher *et al.* (1992) suggested that at constant biomass and short substrate availability, biodegradation rate is directly proportional to the residual pesticide concentration.

Effect of carbon source: Presence of nutrients other then pesticide has a remarkable influence on biodegradation. These nutrients may boost the growth on microbe or damage them being toxic. At 400 mg/L *Pseudomonas* sp. degraded 53.6 % chlorpyrifos in 18 days (Fig. 2). However with the addition of nutrients like glucose and galactose the degradation increased by 21 and 18%, respectively. Yeast extract also positively influenced the degradation and increase the process by 13%. Whereas mannose and starch has minimal effect, their addition increased the chlorpyrifos by 6 and 2%, respectively (Fig. 3). The results of Singh *et al.* (2004) are contradictory with present study, who reported *Enterobacter* strain which stops chlorpyrifos degradation with the addition of glucose and after 1.5 days the degradation process started again. On the other hand, in present study *Pseudomonas* sp. not only carry on chlorpyrifos degradation but boost the process. The elevated biodegradation rate with addition of glucose is a sign that glucose has a critical role in initial growth of *Pseudomonas* sp (Swetha and Phale, 2005). This boosts in biodegradation perhaps due to co-metabolism, where addition of easily metabolized organic matter boost degradation of such compounds which are usually not used as energy and carbon. Earlier findings suggested the use of glucose as co

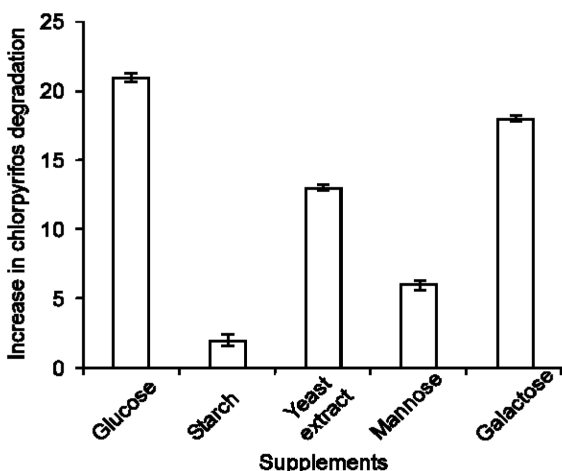

Fig. 3: Effect of added supplements on chlorpyrifos degradation. pH: 7, inoculum density: 10^4 (CFU/mL), incubation: 21 days and chlorpyrifos concentration: 400 (mg/L)

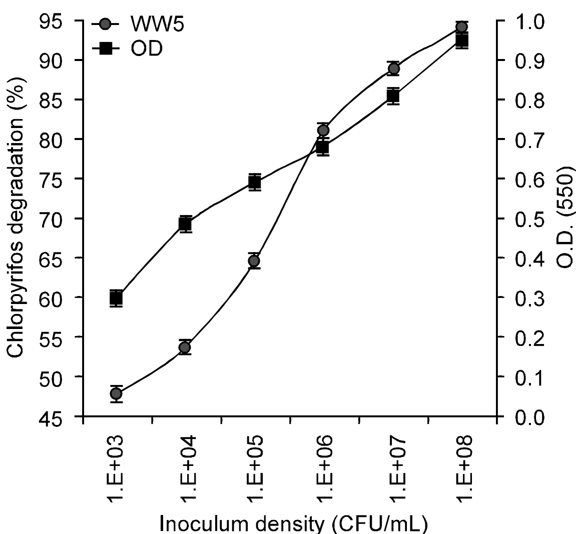

Fig. 4: Effect of inoculum density on chlorpyrifos degradation. pH: 7, incubation: 21 days, chlorpyrifos concentration (400mg/L) and no added supplement

substrate and the process of co-metabolism is widely accepted applications for biodegradation management (Sarkar *et al.*, 2010).

Effect of inoculum density on degradation of chlorpyrifos: Initial inoculum densities of 10^3–10^8 CFU/mL were tested and *Pseudomonas* and it was found that the inoculum density has a direct relationship with chlorpyrifos degradation (Fig. 4). With the highest inoculum density of 10^8 CFU/mL, chlorpyrifos degraded up to 94% within 18 days of incubation with apparently no lag phase. Whereas, low inoculum density (10^3 CFU/mL) shows longer lag phase and degraded maximum of 47%. In general, longer lag phases were observed prior to speedy degradation. At the start of biodegradation process the number of active bacterial population used to be small, longer lag phase represents the time required to maintain the certain significant number of bacterial population. Before that significant number of active bacterial population biodegradation cannot proceed (Anwar *et al.*, 2009). This significant number depends on resistant level of microbial strain and also on the chemical nature of material to be degraded (Fang *et al.*, 2008).

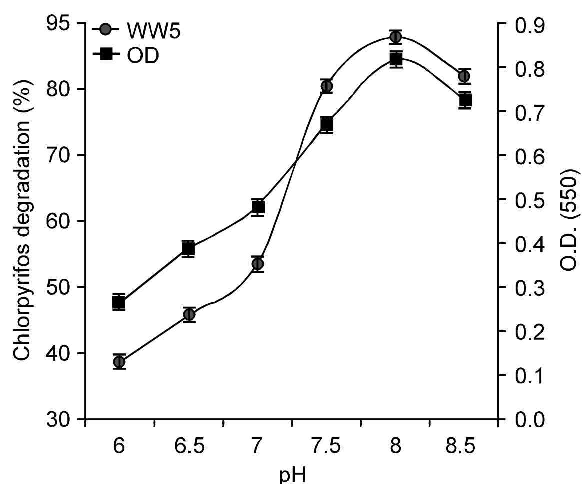

Fig. 5: Effect of pH on chlorpyrifos degradation. Chlorpyrifos concentration: (400mg/L), inoculum density: 10^4 (CFU/mL), incubation: 21 days and no added supplement

Effect of media pH: One of the important abiotic factors that affect the microbial ability towards biodegradation is pH. *Pseudomonas* exhibits degradation at most of the pH ranges (alkaline to acidic) but with varying degree (Fig. 5). Maximum degradation was observed at pH 8. Singh *et al.* (2008) reported rapid chlorpyrifos degradation by an Enterobacter sp at higher pH, while it was significantly slow at low pH. Conversely, Karpouzas and Walker (2000) reported *Pseudomonas putida* (epI and epII) which quickly degraded organophosphate pesticide (ethoprophos) from pH 7.6 to 5.5. In the present study, higher pH shows maximum degradation. Possibly, chlorpyrifos degrading enzymes have optimum activity at high pH (Swetha and Phale, 2005). Present study validate that the isolated *Pseudomonas* sp. could be used for soil restoration and chlorpyrifos degradation.

Conclusions: For the purpose of ecological restoration, bioremediation/biodegradation based techniques are gaining popularity. One of the most vital steps in successful biodegradation, is the selection of resistant and appropriate/competent microbial strains. The most

successful elimination of contaminants may be achieved using inoculants isolated from contaminated environments (where contamination had occurred over years). These indigenous microbe give duel benefit, first they detoxify the contaminant and secondly do not pose any serious threat to other native flora and fauna. Many biotic and abiotic factors significantly influence the process of biodegradation. In conclusion, present results validate the potential of *Pseudomonas* sp. for chlorpyrifos degradation. This strain can be used for bioremediation of contaminated sites.

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