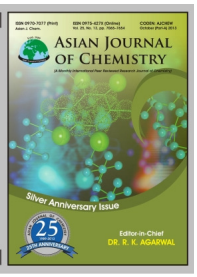
Asian Journal of Chemistry; Vol. 25, No. 17 (2013), 9994-9998

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.15905



# Chlorpyrifos Biodegradation in Laboratory Soil Through Bio-Augmentation and Its Kinetics

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(*Received*: 13 June 2013; *Accepted*: 30 October 2013) AJC-14323

The consumption of pesticides is increasing at an alarming rate in the third world countries including Pakistan and is creating serious environmental and health concerns. Pesticides are often persistent in soils and reach to humans by entering into food chain. Biostimulation and bioaugmentation are considered as most reliable techniques for pesticide remediation, being eco-friendly. The present study deals with the biodegradation of chlorpyrifos in soil. Selected isolate of previous study were tested in soil to explore *in situ* bioremediation possibilities. It was noted that the strain which was potent in liquid media also showed good result in soil. The degradation percentage was 84 by *Klebsiella* sp. Enhancement potential of different organic amendments was studied and the results revealed that farmyard manure has most enhancing effect. Lastly, the biodegradation kinetics in soil was calculated. The kinetic data revealed that *Klebsiella* sp. have the potential for bioremediation and can be used for rapid chlorphyrifos degradation. The present study was thus innovative and successful in providing the eco-friendly solution using indigenous bacteria for countering chlorphyrifos pollution. This strain can be used for soil and ecological restoration.

**Key Words: Bioaugmentation, Biodegradation, Chlorpyrifos, Eco-restoration, Kinetics.**

## INTRODUCTION

Pesticides are in extensive use for the last 50 years. The negative side of pesticides was highlighted after 2 decade of their use and was connected to many health disorders, especially cancer, poisoning and deaths[1]. These pesticides ultimately enter in soil and mostly adversely effects diversity of soil microflora and microfauna. This finally maneuvers soil fecundity, plant growth and cause a serious hazard to agricultural sustainability[2]. Soil microbial diversity is regarded as one of the most important indicator of ecosystem health. Therefore, the impact of pesticides on soil microbial diversity has raised considerable public concern[3].

Chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is a broad-spectrum organophosphate insecticide and is commonly used on cotton, grain, nut, fruit and vegetables, lawns and ornamental plants to control pests. Because of its intensive use, a wide range of terrestrial ecosystems may be contaminated with chlorpyrifos. There is a need to evaluate its environmental behavior and effects. The dissipation, adsorption, leaching, photolysis and biodegradation of chlorpyrifos in soil ecosystems have been extensively investigated[4]. The half-life of chlorpyrifos in soil varies greatly from less than 1 day to more than 100 days depending on the soil type, soil microorganisms and climatic condition. Chlorpyrifos effects microbial biomass, populations, respiration, enzymatic actions, and carbon/nitrogen cycling. 10-50 mg $kg^{-1}$ of chlorphyrifos may reduce microbial biomass[5] up to 25-50 %. Nitrogen mineralization in the loamy sand and sandy loam also significantly inhibit by chlorpyrifos application. Shan *et al.*[2] also indicated that soil bacterial, fungal and actinomycetes populations were inhibited by chlorpyrifos at a concentration of 10 mg $kg^{-1}$. However, little information is available on the impact of chlorpyrifos on soil microbial diversity.

In Pakistan and other developing countries, pesticide problem is also enhanced by the lack of disposal procedures and use of expired agrochemicals. In totality, all these problems contribute agrochemical residues in soil, drinking water, ground/surface water, soft drinks and registered mineral water. In Pakistan, due to lack of analysis and laboratory facilities the data regarding pesticide contamination in drinking water is inadequate[6]. In the present study, *Klebsiella* sp. was used to investigate its potential role in eco-restoration and its kinetics.

## EXPERIMENTAL

Analytical grade (Sigma-Aldrich, Merck, Oxides or BDH) chemicals were used throughout the study. Chlorpyrifos (95 %) was obtained from Pak China Chemicals, Lahore. Commercial grade chlorpyrifos was purchased from local market. All the growth media, glassware, culture loops and needles were sterilized before experimentation.

**Sampling:** Soil samples were collected from cotton growing fields of Multan, Bahawalpur, Bhawalnagar and Rehim Yar Khan regions. These soils are under extensive pesticide spray over the years. Top soil up to 10 cm deep was collected by the standard methods[7]. All the samples were collected in sterile glass bottles. These glass bottles were transported in ice box and were stored at 4 °C in order to limit any physico-chemical change.

**Enrichment, isolation and selection of microbial strains:** Bacterial strains capable of degrading chlorpyrifos were isolated from soil samples in minimal salt medium (MSM) at 6.8-7.0 pH in previous study[8]. For this research work the same strain was used for soil experimentation.

**Culture maintenance:** Selected isolates were aseptically picked up and transferred to nutrient agar slants. The slants were then incubated at 37 °C for 48 h for maximum growth and then stored at 4 °C. Sub culturing was carried out after every 2 weeks

**Inoculum preparation:** A loop of bacteria was aseptically transferred to 100 mL sterile nutrient broth in a 500 mL flask. The flask was incubated for 24 h on a rotator shaker (200 rpm) at 37 °C. This bacterial suspension was used to prepare inoculum for further study. Required size of the inoculums was attained by quantifying the cell number using haemocyto-meter[9].

**Bioaugmentation experiment:** Bioaugmentation experimentation was conducted in order to investigate the biodegradation potential of selected strain in soil. All the experimentations were run in triplicate. To study the effect of pesticide concentration on degradation of chlorpyrifos, measured concentration of chlorpyrifos was mixed with 100 g sterile soil and quantified inoculum in a glass beaker. Same setup without inoculum was kept as control. After every 24 h, soil samples were withdrawn and pesticide residues were extracted and analyzed. Parameters investigated were pesticide concentration, pH range, temperature ranges, carbon sources and inoculum size.

**Kinetics study:** Michaelis-Menten model was used to calculate biodegradation kinetic constants[10]. The general form of Michaelis-Menten kinetic relation is:

$$\frac{dS}{dt} = -V_{max}\frac{S}{S + K_S} \quad (1)$$

where; S = concentration of substrate, $V_{max}$ = maximum biodegradation rate and $K_s$ = half saturation constant.

**Extraction of chlorpyrifos:** 20 g of soil sample was mixed with 20 mL distilled $H_2O$ and 50 mL acetone in a flask and transferred to rotator shaker for shaking up to 2 h at 150 rpm[11]. The mixture of soil, distilled water and acetone was filtered under suction. 25 mL acetone was used for rewashing of filter cake. Re-washing was done thrice. Filtrates form all washing were collected in flask and it was left over laminar air flow table for 2 h to evaporate acetone. The remaining material was then transferred to separating funnel and equal volume of supernatant and dichloromethane were mixed and organic layer of dichloromethane was collected. Dichloromethane was evaporated under nitrogen at room temperature. For the removal of any particle, 0.45 μm diameter flouropore™ filter membrane was used. Residues were filtered after dissolving in acetonitrile[11].

**HPLC analysis:** Varian HPLC (equipped with a ternary gradient pump, UV detector, electric sample valve, column oven and $C_{18}$ reversed-phase column) was used for pesticide analysis using mobile phase of methanol:water (85:15, v/v). HPLC conditions were set as follows; 20 μL sample volume, 1 mL $min^{-1}$ flow rate, 15 min retention time and 290 nm wavelength[12].

## RESULTS AND DISCUSSION

Toxic chemicals of organic and inorganic nature are the serious threat to human health. Prevention, removal and conversion (to non-toxic states) of these toxic materials are challenging. Numbers of physio-chemical techniques are available but they are much expensive, target non-specifically and may initiate secondary contamination. For that reason, eco-friendly abetment techniques are the need of an hour[13]. Bioremediation is considered as less expensive, less laborious, eco-friendly and efficient. For this bioremediation is gaining popularity for environmental cleanup applications. The present study also deals with the enhanced bioremediation of chlorpyrifos in soil. One of the most important factors in enhanced bioremediation is the presence of resistant microbial agent, which may bear the high level of toxicity[14].

**Microbial biodegradation of pesticide:** *Klebsiella* sp. is a versatile genus and can successful degrade pyrene, propionitrile, azo dyes, organophosphate pesticide and present study also supported their immense biodegradation diversity[8]. Triumphant bioremediation is dependent on use of potent microbe. For bioremediation, microbes must be resistant against high concentration of pollutant and its intermediate metabolites. The present study investigated the biodegradation potential of isolated *Klebsiella* sp. and found it effective. Number of isolates has been reported by many researchers, which were able to degrade chlorphyrifos and other pesticides. This include *Aspergillus* sp. *Trichoderma*, *Pseudomonas*, *Agrobacterium*, *Pseudomonas putida*, *Bacillus pumilus*, *Klebsiella* sp., *Serratia marcescens*, *Pseudomonas fluorescence*, *Enterobacter* sp., *Aeromonas* sp. and *Chlorella vulgaris*. The abilities of these microbes to degrade chlorphyrifos in natural conditions are still unconfirmed[15]. Those microbes which cannot compete with soil microflora are not considered potential candidate for *in situ* bioremediation[16].

**Impact of chlorphyrifos concentration:** Fig. 1 depicts the rate of degradation by *Klebsiella* sp. at different concentrations and exhibited tolerance up to 70 g $Kg^{-1}$ of chlorphyrifos. The maximum chlorphyrifos biodegradation was observed with low concentration. This shows that the chlorphyrifos concentration has inverse relationship with rate of biodegradation. At high concentration longer lag phase was

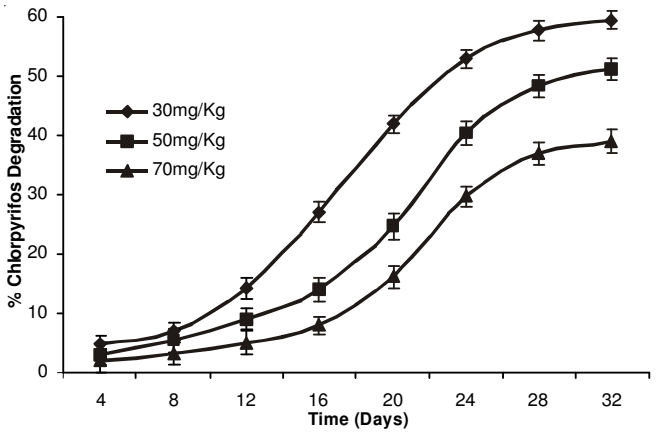


Fig. 1. Degradation of chlorpyrifos at different initial concentrations

observed, which last for 12-16 days. At 70 g $Kg^{-1}$ and 50 g $Kg^{-1}$ only 8 and 9 % degradation was observed during lag phases, respectively. This lag phase represents the time duration by which microbes adapt themselves according to the new environment and maintain their sufficient number which is required for rapid biodegradation. The stationary phase started by the 28th day, which represents the maximum degradation. The maximum degradation at 70 g $Kg^{-1}$ was 39 %. This wide tolerance range against chlorphyrifos concentration is very useful for ecological restoration programs. Hua *et al.*[17] conducted chlorphyrifos degradation in soil with the initial concentration of 4, 8 and 12 g $Kg^{-1}$ and reported biodegradation up to 83 %, 81.6 % and 79.5 %, respectively in 35 days. Shan *et al.*[2] reported sharp inhibitory effect at 10 g $Kg^{-1}$. Singh *et al.*[16] concluded that *Entrobactor* sp. can survive the initial concentration of 35 g $Kg^{-1}$. Similarly, *Bacillus pumilus* C2A1 have ability to withstand 25-50 g $Kg^{-1}$ of chlorphyrifos in soil. This strain exhibits 97 % degradation in 45 days and its presence also decreases the translocation of chlorphyrifos in plant (rye grass) tissues especially shoot and root[18].

**Impact of temperature:** Fig. 2 represents the chlorphyrifos degradation pattern by *Klebsiella* sp. at different temperatures. All the temperature ranges differ significantly from one another. The optimum growth and degradation temperature was 35 °C, bearing maximum degradation up to 55 %. On the other side minimum degradation was at 40 °C, which was up to 39 %. At 35 °C *Klebsiella* sp. shows shorter lag phase and slow degradation rate till 12th days, after which the rapid increase was observed. The difference between maximum and minimum degradation at 35 °C and 40 °C was 41 %, which emphasize the significance of temperature in biodegradation process. The results also highlight the temperature tolerance range of *Klebsiella* sp. This wide temperature tolerance range is extremely useful for field application. So microbes having wide temperature tolerance range, are more useful as compared to the microbes with narrow temperature range. Sarkar *et al.*[3] was able to isolate *Pseudomonas* sp. from tea rhizosphere, which showed 69 % degradation of propargite (insecticide) at 30-35 °C. Similarly *Enterobacter* sp. show optimum temperature of 35 °C. Degradation ability of *Enterobacter* sp. did not effected significantly within the range of 25-40 °C, but further decrease (below 15 °C) or increase (above 50 °C) in temperature drastically reduced the biodegradation[16]. The temperature 37 °C has been recommended as optimum temperature for *Agrobacterium*, *Bacillus cereus* and *Pseudomonas aeruginosa*[10]. Similarly, biodegradation study by Anwar *et al.*[19] reported 90 % chlorphyrifos degradation (300 g $L^{-1}$) by *Bacillus* sp. at 37 °C. Abboud *et al.*[20] reported 90 % degradation of alkylbenzo-sulfonate and sodium dodecyl sulfate by *Acinetobactor calcoaceticus* and *Pantoea agglomerans* at 30 °C. *Verticillium* sp. (a fungal strain) capable of degrading chlorphyrifos, showed maximum efficiency at 35 °C, which was 1.12 times faster than 20 °C. Experimentation revealed that *Bacillus cereus* worked maximum at 30 °C and showed 78 % degradation[21].

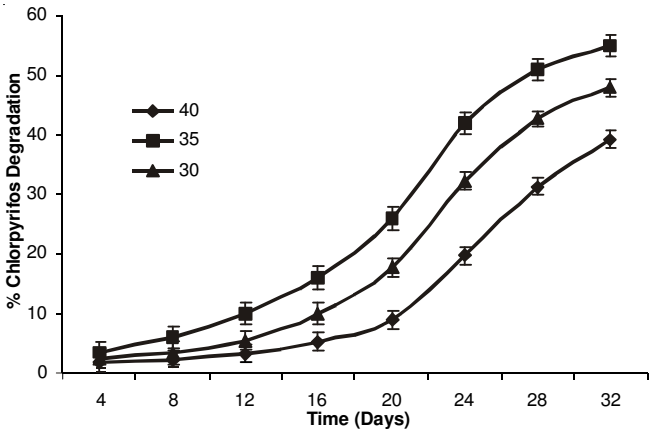


Fig. 2. Degradation of chlorpyrifos at different temperature range

**Impact of pH:** Degradation of chlorphyrifos at all pH ranges is non-significant in the start of experimentation (4th day), but it increased with time (Fig. 3). Maximum degradation in 32 days at 8.5, 8, 7.5 and 7 pH was 67, 73, 60 and 55 %, respectively. Degradation reaches at almost stationary phase at 28th day. Optimum pH for *Klebsiella* sp. was 8, increase or decrease from this pH negatively effects the chlorphyrifos degradation. *Klebsiella* sp. significantly degraded chlorphyrifos at all pH and almost same pattern was shown. These results conclude that the change in pH affects the degradation rate and percentage, but it will continue to proceed and do not stops with the slight disturbance in the environment. Results of the other studies also conform this trend[20]. This wide pH range sounds very significant. As the environmental conditions keeps on changing and hence microbes with wide tolerance range have better chance of survival. Singh[22] reported rapid chlorpyrifos degradation by an *Enterobacter* sp. at higher pH, while it was significantly slow at low pH. Conversely, *Pseudomonas putida* quickly degraded ethoprophos (organophosphate pesticide) from pH 7.6 to 5.5. *Bacillus cereus* demonstrated optimum pH of 7, but pH 6 and 8 also show significant similar result compared to pH 7. However pH greater than 8 and less than 6 significantly inhibit chlorphyrifos degradation[21]. Beside bacteria, fungal strains can also be used for biodegradation of chlorphyrifos and ecological restoration. Fang *et al.*[12] isolated *Verticillium* sp. from contaminated soils. This fungal strain was more efficient at pH 7. At pH 5 and 9 it exhibited 1.12 and 1.04 times slower degradation rate.

**Impact of carbon source:** To investigate the potential role of soil amendments in biodegradation process, 3 organic

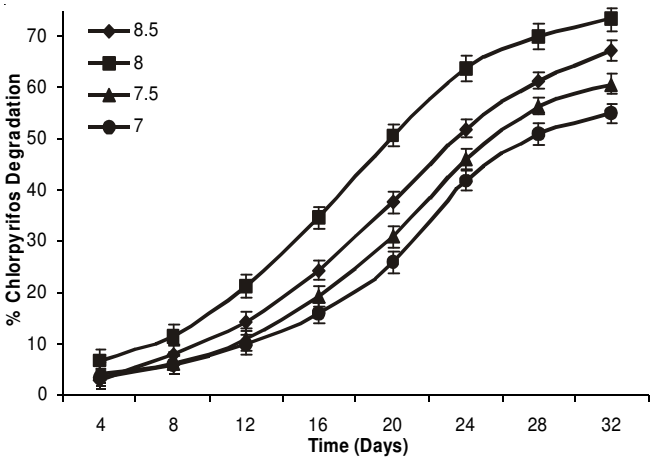


Fig. 3. Degradation of chlorpyrifos at different pH

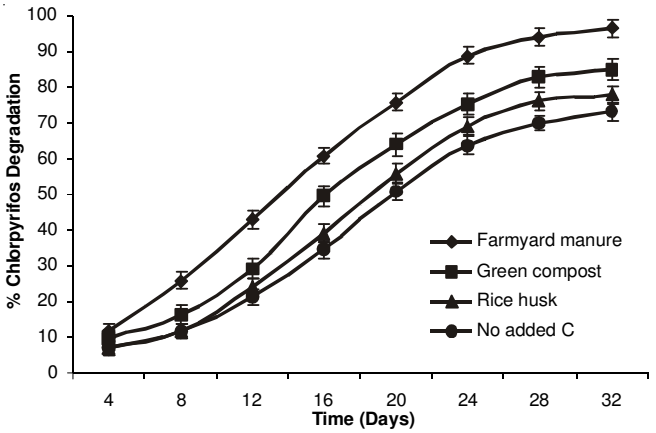


Fig. 4. Degradation of chlorpyrifos in different carbon sources

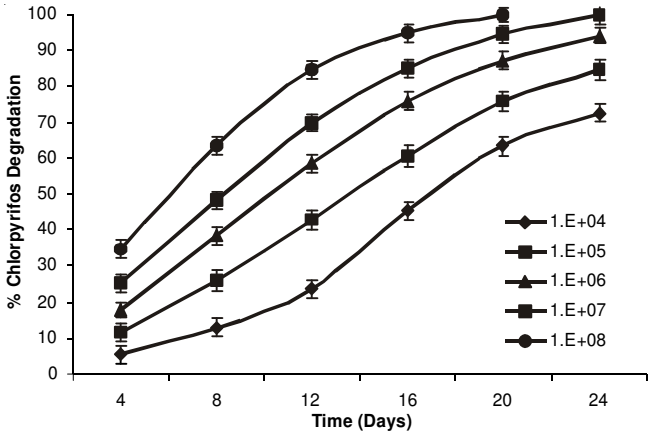


Fig. 5. Degradation of chlorpyrifos at different inoculum size

materials were tested, *i.e*. farmyard manure, green compost and rice husk. From the results (Fig. 4) it can be stated that the organic amendments can increase the ability of *Klebsiella* sp. towards biodegradation of chlorphyrifos. In presence of farmyard manure, rapid or enhanced biodegradation started from the start of the experiment and attain stationary phase at day 28. It exhibited maximum degradation of 96.7 % in 32 days. Whereas, in presence of green compost first 8 days showed slow degradation and then afterward rapid degradation started. In presence of green compost maximum degradation of 85 % was achieved. Rice husk showed least enhancing effect, it reached to the stationary phase at almost 24$^{th}$ day. Beyond 24$^{th}$ day, degradation rate was not much prominent. In compassion to no organic amendment, addition of farmyard manure, green compost and rice husk, enhanced biodegradation of chlorphyrifos up to 32, 16.4 and 7%, respectively. Organic amendments improve soil fertility by stimulating the soil micro flora. Therefore, many studies has reported the usefulness of different organic amendments like, farmyard manure, public green compost, urban solid waste, coconut husk, rice husk, poultry liter, sheep manure, nut shells, mushroom spent and cow manure[23]. These organic wastes contain humic acid and fulvic acid, which alter the bioavailability of chlorphyrifos to microbes. It was suggested that those organic amendments which contain high humic acid favors rapid chlorphyrifos degradation. However, fulvic acid play minimum role in bioremediation of chlorphyrifos in contaminated soils. Investigation in changes of microbial community size and composition with addition of nitrogen and green manure revealed that green manure increase microbial biomass[24]. It is assumed that individual production practices exert bad effects on microbial biomass and total carbon in soils. Labile carbon in soil can be transformed into stable pools by reducing tillage and increasing organic carbon contribution[25]. Pesticide transport in the presence of organic amendments is still unclear. On one hand, high organic content favors the retention of pesticides in soil. On the other hand, high dissolve organic matter may assist their mobility and transport. However, many authors are still unable to develop strong relationship between pesticide transportation and amount of organic matter. This uncertainty may be because of the wide structural variation of organic material used[26].

**Impact of inoculum density:** Fig. 5 shows the effect of inoculum density on chlorphyrifos degradation by *Klebsiella* sp. All the inoculum treatments show almost the same pattern of biodegradation. Lag phases were not observed in any treatment. However, slight slow rate was shown by *Klebsiella* sp. at $10^4$ (CFU $g^{-1}$). At $10^8$ and $10^7$ (CFU $g^{-1}$) the degradation rate was very steep and within 12 days degradation reached up to 84.7 and 70 %, respectively. Addition of high inoculum reduced the total time duration. With $10^5$ (CFU $g^{-1}$) 96.7 % degradation was achieved in 32 days, where as inoculum size of $10^8$ (CFU $g^{-1}$) attained 100 % degradation in 20 days. Similarly, $10^7$ (CFU $g^{-1}$) maintain 100 % degradation in 24 days. These results indicated that *Klebsiella* has the potential of rapid degradation, which can be attained by adjusting different environmental conditions. Maximum degradation in 24 days with $10^4$, $10^5$, $10^6$, $10^7$, $10^8$ (CFU $g^{-1}$) was 72.7, 84.7, 94.2, 100 and 100 %, respectively. Size of the inoculum plays very promising role towards biodegradation in soil. Liang *et al.*[27] reported *Diaphorobacter* sp. GS-1, capable of degrading chlorpyrifos, triazophos and 1-phenyl-3-hydroxy-1,2,4-triazole up to 100 %, 95 % and 100 %, respectively, at inoculum density of $10^7$ CFU $g^{-1}$ in 21 days. Strain B-14 successfully degraded 100 % with inoculum size of $10^6$ cells $g^{-1}$. However this strain was not successful in degrading TCP. Strain Dsp-2 showed 98 % degradation in 30 days with 100 g $Kg^{-1}$ (initial concentration)[13]. Similarly, in lab scale soil experimentation of 30 days, *Cupriavidus* sp. DT-1. degraded 100 % chlorphyrifos and 94.3 % of TCP with $10^6$ cells $g^{-1}$ (initial inoculum)[28].

**Kinetics study:** Chemical reaction catalyzed by enzymes show hyperbolic curve between substrate concentration and reaction rate. When the substrate concentration is low the enzymes active sites are vacant and thus has the ability to accommodate more substrate. For this reason, at low substrate concentration the rate of reaction increases with the increase in substrate concentration. "$V_{max}$" is the maximum reaction rate at enzyme saturation point. Correlation among reaction rate and substrate concentration is dependent on the enzyme affinity for substrate. This correlation is expressed as "$K_m$ (Michaelis constant)", an inverse of the affinity. For calculation and practical purposes, "$K_s$" is used instead of "$K_m$"[10]. $K_s$ is the concentration of substrate which permits the enzyme to achieve half $V_{max}$. Apparently, enzyme having high value of $K_s$ has low substrate affinity and subsequently it need high substrate concentration to attain $V_{max}$[29]. $K_s$ and $V_{max}$ are important in predicting the rate of formation of product and whether or not substrate availability affect reaction rate. Those enzymes which have low value of $K_s$ are usually saturated and work at relatively constant rate. Such enzymes do not get affected with narrow ranges in substrate concentration. On the other hand, enzymes having high $K_s$ are not physiologically saturated. As a consequence, changes in substrate concentration affect enzyme activity and rate of reaction. $V_{max}$ and $K_s$ are calculated by measuring the enzyme activity at different substrate concentration. The result will give hyperbolic curve, when substrate concentration [S] is plotted against reaction rate [V][30].

The data of biodegradation kinetics in soil is represented in Table-1. The straightness of the Hanes plot is represented by the $R^2$ (0.9948). The ratio of $V_{max}/K_s$ is considered more useful tool in predicting the efficiency of the isolate toward biodegradation. The range of this ratio was 0.0481. Maya *et al.*[10] reported $K_m$ and $V_{max}$ for chlorphyrifos and TCP for *Bacillus, Pseudomonas* and *Agrobacterium*. The range of $K_m$ for chlorphyrifos was from 97-142.3 mg $L^{-1}$ and 103.09-148.8 mg $L^{-1}$ for TCP. The $V_{max}$ varied from 7.4-12.1 mg $L^{-1}$ and 14.9-21.2 mg $L^{-1}$ for chlorphyrifos and TCP, respectively. Fang *et al.*[12] calculated reaction rate ($V_{max}$) as 12.171 and $R^2$ as 0.9870 for *Verticillium* sp.

TABLE-1
KINETIC DATA OF CHLORPYRIFOS BIODEGRADATION IN SOIL

| Isolate | $V_{max}$ (mg $L^{-1}$ $D^{-1}$) | $K_s$ (mg $L^{-1}$) | $V_{max}$ / $K_s$ | $R^2$ |
|---|---|---|---|---|
| *Klebsiella* sp. | 8.4746 | 176.1473 | 0.0481 | 0.9948 |

## Conclusion

Excessive use of pesticides is creating 2 main problems. At first they are damaging the soil microbial composition and secondly reducing the soil fertility. Bioremediation is earning popularity as the pesticide pollution is increasing at an alarming rate in the agricultural sector, posing serious threats to humans and the pristine ecosystems. The present study was thus innovative and highly successful as it provided the eco-friendly solution using indigenous bacteria against chlorphyrifos pollution. This strain can be used for soil and ecological restoration and habitat improvement.

## ACKNOWLEDGEMENTS

The authors are grateful to Government College University for providing funding and laboratory infrastructure. Thanks are also due to Cotton Research Institute Multan for helping in field work. Special thanks to Pakistan Council for Scientific and Industrial Research for technical assistant.

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