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RESTORATION OF PESTICIDE CONTAMINATED AGRICULTURAL SOIL THROUGH BIO-AUGMENTATION AND ITS KINETICS

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Excessive use of pesticides is evident in third world countries and Pakistan is not the exception to this. Most of these pesticides are persistent and they remain present in ecosystem as active ingredient for longer duration. Their presence cause serious damage to micro flora and fauna, thus disturbing the functioning of ecosystems. After absorbance, they enter into food chain and tend to bio-accumulate. In the present situation, bio-augmentation and bio-stimulation are considered most safe techniques to clean pesticide contaminated soils. These techniques are natural, cost effective, do not produce toxic by products and easy to handle. The present study was designed to degrade chlorpyrifos (CP) in laboratory soil using *Pseudomonas* sp., which was isolated from industrial drain. This strain showed promising results in both liquid medium and soil. This strain degraded up to 60mg of CP per one Kg of soil. Among different organic amendments, farm yard manure was most effective and showed highly significant results. Kinetic analysis and its data also provide strong evidence of success. Kinetic parameters were as follow; $R^2 = 0.9917$, $K_s = 159.0271$, $V_{max} = 9.64$ and $V_{max}/K_s = 0.0606$. This research is successful as it provided indigenous solution to a environmental problem.

Keywords: Eco-restoration, bioremediation, bio-augmentation, pesticides, chlorpyrifos, kinetics

INTRODUCTION

History of cotton farming dates back to South America and Indus valley civilizations. Before mid twenty century, different techniques like crop rotation, agricultural management, crop density, tillage practices and pest cycle were used to control cotton pests (EJF, 2007). Different neurotoxic chemicals were invented soon after World War II. During the last fifty to sixty years, these toxic chemicals were extensively used to protect cash crops (Zia *et al*., 2008). These agrochemicals were preferred by farmers, because they are easy to use, long lasting effect, cheap and less laborious. Like other developing countries use of pesticides in Pakistan was a new incident. For example, in Pakistan, 5-10% of cotton growing area was receiving pesticides up till 1983 but it increased up to 98% in 1991 (Tariq *et al*., 2007). Reporting of harmful side of pesticides in term of human health was started after two decades of their use. It is now well documented that these chemicals are associated with different diseases especially cancer. Pesticides with well known toxicity are ban in developed countries but are still in use in developing countries (Sankararamkrishnan *et al*., 2005). True picture of pesticide poisoning is not available due to unavailability of insufficient and reliable data (Lozowicka *et al*., 2014). Even strict rule and regulations in developed countries are not able to protect occupational exposure. To study contamination of pesticides in ground water only a single national level study was carried out in Pakistan (PPSGDP, 2002). Fate of pesticides in environment depends on multiple factors, like physio-chemical property of the compound, soil conditions, abiotic/biotic conditions and application practices. Pesticides make strong affinity with soil particles, sediments and roots when applied in fields (Gebremariam *et al*., 2012). With the passage of time, these compounds degrade/decompose in to different metabolites (usually become more soluble) or volatilized (Marino *et al*., 2002). Thus, the pesticides become available in three states i.e. liquid, solid and vapors. Their distribution mainly depends on different physio-chemical property of active compounds (Burauel and Bassmann, 2005). Complete or incomplete mineralization of pesticides occurs as a result of decomposition by microbes in soil (Zhang *et al*., 2012). About 80% of total agrochemicals are using for cotton crops, about 0.1% of which reached to targeted pests (Tariq, 2005). During last twenty years in Pakistan, about 1169% increase has been observed in the use of agrochemicals (Tariq *et al*., 2007). The time required (weeks or months) for decomposition of the agrochemicals by microbes and complexity of the process are the main constraints which is slowing down the technology to develop viable method of remediation. It is clear that detailed studies of biodegradation

processes are required to develop effective method to solve the issue of persistent organic pollutant. The present study was designed to degrade chlorpyrifos (pesticide) in soil, using resistant bacterial specie. This resistant bacterial specie was isolated from industrial drain carrying waste-water from pesticide manufacturing unit.

MATERIALS AND METHODS

***Bio-augmentation experiment*:** During previous study, bacterial strains able to degrade chlorpyrifos were isolated from wastewater collected from drains receiving wastewater from different industries (Farhan *et al*., 2012). The same strain was used for soil experimentation in this study. Haemocytometer was used to make known size of inoculums. Sterile glass beakers were used to mix measured quantities of chlorpyrifos, the inoculums and 100g sterile soil. Control was prepared by the same steps without inoculum. From the mixture, soil samples were withdrawn after every 24 hours for the extraction and analysis of pesticide residues. Five parameters including pesticide concentration, temperature range, pH range, inoculums size and carbon source were analyzed. All the experiments were design in triplicate.

***Kinetics study*:** Biodegradation kinetic constant was calculated by using Michaelis-Menten model (Maya *et al*., 2011). The common form of Michaelis-Menten kinetic relation is as under:

$$\frac{dS}{dt} = -V_{max}\frac{S}{S + K_s} \qquad \text{.....eq. 1}$$

Where; 'K_s' is the half saturation constant, 'V_{max}' is maximum biodegradation rate and 'S' is concentration of substrate

***Extraction of chlorpyrifos*:** 20 ml distilled water was added in 50 ml acetone in a flask, then 20g soil sample was mixed in the solution and the flask was shake by rotator shaker up to two hours at 150 rpm (XG). Filtration of the mixture was carried out by suction and filter cake was rewashed by 25ml acetone, thrice. For the evaporation of acetone from the filtrate, it was left over laminar air flow table for two hours. Remaining material was mixed with equal volume of supernatant and dichloromethane in a separating funnel and layer of dichloromethane was collected. Under nitrogen at room temperature dichloromethane was evaporated. Flouropore^{TM} filter membrane with 0.45 µm diameter were use to ensure the removal of any particle. Residues were dissolved in acetonitrile for filtration (Fang *et al*., 2008).

***HPLC analysis*:** Varian HPLC equipped with UV detector, column oven and ternary gradient pump with C18 reversed phase column was used. Methanol:water (85:15v/v) was use as liquid phase at flow 1mLmin^{-1}, retention time 15 minute and wavelength 290nm. Sample size was 20ml for each analysis.

RESULTS AND DISCUSSION

***Biodegradation against varying CP concentrations*:** Concentration range from 40-80mgKg^{-1} was examined for degradation by *Pseudomonas* sp (Fig. 1). Lag phase was not detected at 40mgKg^{-1} and high degradation rate was observed up to day 21. After that the reaction rate reaches at equilibrium. However, prominent lag phase was observed at higher concentration of 60 and 80 mgKg^{-1}. The maximum biodegradation of CP was 69% at 40mgKg^{-1}, 60% at 60 mgKg^{-1} and 45% at 80 mgKg^{-1}. Control set up did not showed any significant degradation. Results revealed that the degradation rate varies significantly as the concentration increases but the good point is that *Pseudomonas* sp. was resistant at high CP concentration. Concentration higher than 80mgKg^{-1} killed this strain and the whole degradation process stopped. For this reason, further all experimentation was conducted at 80mgKg^{-1}. Similar findings were observed by Hue *et al*. (2009) where 79.5% CP was degraded at 12mgKg^{-1} in 35 days. Degradation was high up to 83% and 81.6% at 4 and 8mgKg^{-1}, respectively. Contradictory to the above finding, inhibition of CP degradation at 10mgKg^{-1} was also observed (Shan *et al*., 2006). *Entrobactor* sp. capable of tolerating 35mgKg^{-1} of CP was also reported (Singh *et al*., 2006). CP degradation of 97% was reported by *Bacillus pumilus* having resistance up to 50mgKg^{-1}. Pot experimentation revealed that this strain reduce the CP translocation in root and stem of ryegrass (Ahmad *et al*., 2012).

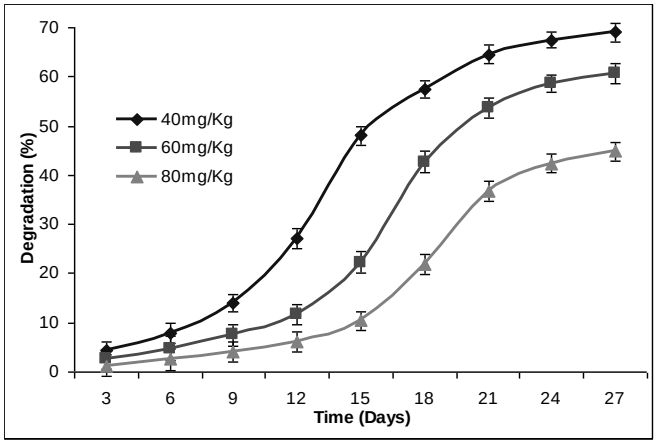

Figure 1. Biodegradation response of *Pseudomonas* sp. at different CP concentrations.

***Biodegradation against varying temperature*:** Temperature tolerance was tested by conducting experiments at 30-40°C (Fig. 2). *Pseudomonas* sp. was tolerant up to 40°C but maximum biodegradation occurred at 35°C (50%). Lag phases varies significantly with temperature, it was 12days at 40°C, 9days at 35°C, and 13days at 30°C. Results revealed that other than 35°C, all temperature shows lengthy lag phase.

Difference between highest and lowest CP biodegradation by *Pseudomonas* sp. was 28.2% (highly significant). Studies recommend that the presence of microbe capable of wide temperature tolerance is more beneficial compared to narrow temperature tolerance. Such microbes can be utilized in wider geographical areas with varying climatic conditions (Cycon *et al*., 2009; Zhang *et al*., 2012).

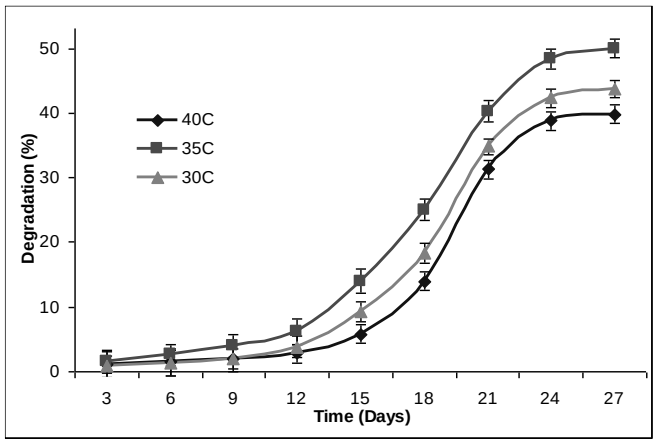

Figure 2. Biodegradation response of *Pseudomonas* sp. at different temperature.

***Biodegradation at varying pH*:** pH plays a vital role in any biochemical reaction. pH was optimized to increase the CP biodegradation by *Pseudomonas* sp. pH range tested was from 7 to 8.5. Maximum degradation was observed at 8 pH and minimum degradation was observed at 7 pH (Fig. 3). pH above 8 do not significantly increase CP degradation. Lag phase also varies significantly with pH. The minimum lag phase of 6 days was observed at pH 8, whereas longer lag phase of 9 days was observed at 8.5 and 7.5 pH. Beyond stationary phase arise where no increase in degradation was observed, after which decline phase appears. Biodegradation of CP varies significantly at different pH. Maximum degradation of 65% was observed at pH 8 and least degradation (50%) was at pH 7. Others studies also supported these results, which indicated that the microbes having tolerance of varying pH are more use full for different soils (Cycon *et al*., 2009; *Zhang et al*., 2012).

***Biodegradation against varying carbon source*:** Presence or absence greatly influences the ability of microbe to degrade pesticides. Carbon sources may co-metabolize and provide energy to the microbes or they may hinder their growth. In present study, results in presence of farmyard manure are quite promising. *Pseudomonas* sp. showed rapid growth and no lag phase was observed in farmyard manure, where as, other carbon sources exhibited varying length of lag phase (Fig. 4). Other carbon sources include rice husk and green compost. Maximum degradation (88%) was observed with farmyard manure and least degradation (73%) was observed with rice husk.

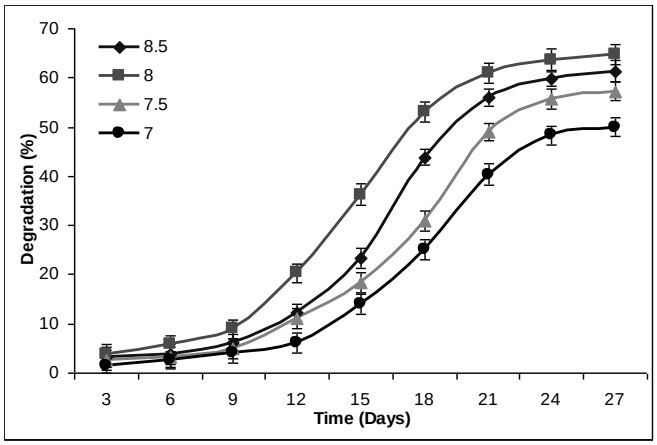

Figure 3. Biodegradation response of *Pseudomonas* sp. at different pH.

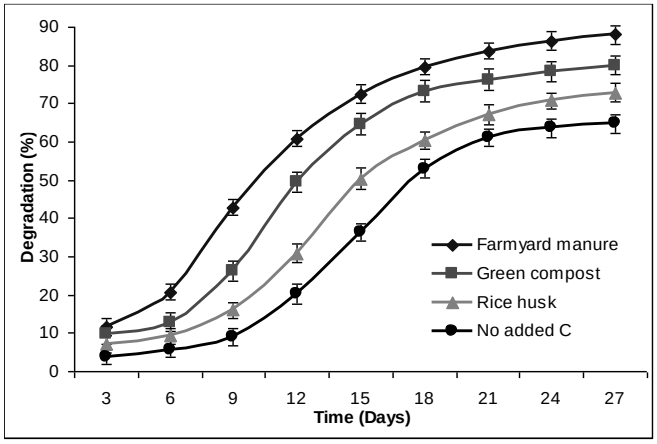

Figure 4. Biodegradation response of *Pseudomonas* sp. at different carbon source.

All the result was significantly different from one another. Start of stationary phase was also variable in all experimental setup and in dependent of carbon sources. In green compost, stationary phase starts at 24th day while in rice husk it started at 21st day. When compared with control set up, 12%, 23% and 35% increase in degradation was observed by rice husk, green compost and farmyard manure. Effectiveness order of all tested materials is as follows;

Farmyard manure > green compost > rice husk.

One possible reason of different effectiveness of different materials is their ability to alter/hinder bioavailability. These organic materials may consist of fulvic and humic acid. Presence of high concentration of humic acid facilitates degradation process but fulvic acid does not speed up the degradation (Tejada *et al*., 2011). Amount of carbon (total) in soils and active microbial biomass depends on the bad crop production practices. Fate of pesticide in presence of carbon sources is still not clear. At one time high carbon (organic) content cause pesticide retention while at other time it favors

transport (Wang *et al*., 2011). Many authors are on the view that this indecision is because of broad and complex structural/chemical nature of supplies (Fenoll *et al*., 2011).

***Biodegradation against varying inoculum size*:** As a general rule, presence of more microbes causes rapid degradation. To optimize the Inoculum size, different ranges were tested (Fig. 5). No lag phase was observed at inoculum size higher than 10^4cfuml^{-1}. This strain showed 100% degradation in 18 days with inoculum density of 10^8cfuml^{-1}. Lack of lag phase is the positive indication that the strain may be used in-situ. Contrary to the above results, inoculum density of 10^3 cfug^{-1} is least useful in initiating degradation. These results are also supported by Ahmad *et al*. (2012). *Diaphorobacter* sp. GS-I degraded 100% of CP in 21 days at 10^7 cfug-1. This strain also degraded 1-phenyl-3-hydroxy-1,2,4-triazole and triazophos up to 99.5% and 95%, respectively (Liang *et al*., 2011). *Cupriavidus* sp. DT-1 showed 100% of CP degradation and 94% of TCP degradation in 30 days soil experimentation having 10^6 cfug^{-1} (Lu *et al*., 2013).

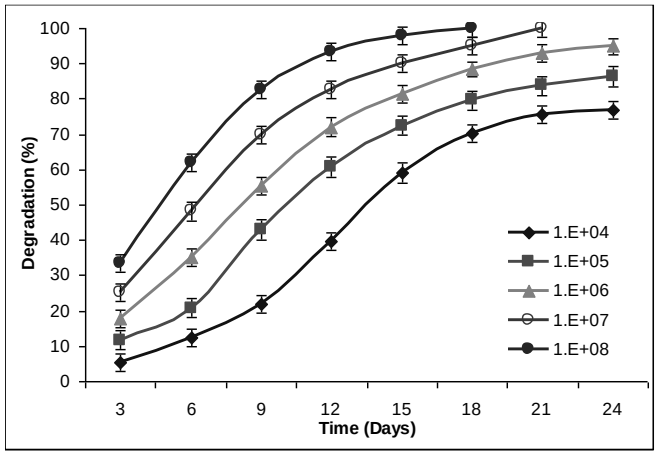

Figure 5. Biodegradation response of *Pseudomonas* sp. at different inoculum size.

***Kinetic study*:** Enzymatic reactions generates hyperbolic curve when plotted between rate of reaction and substrate concentration. Rate of reaction is directly proportional to low substrate concentration, this may be due to the availability of active sites. As the substrate concentration increases the reaction rate tends to become constant. 'V_{max}' is the saturation point of enzyme where rate of reaction is constant. 'K_m' is the 'Michaelis constant' which shows enzyme efficient. Practically, 'K_m' is replaced by 'K_s' and many authors prefer 'K_s' for calculation purpose (*Maya et al* 2011). Substrate concentration at which the enzyme attains '½ V_{max}' is called 'K_s'. Generally, high K_s value means low affinity between enzyme and substrate and V_{max} will be attained only at high concentration of substrate. Value of R^2 (0.9917) represent the Hanes plot, value more near to '1' means more straightness (Fig. 6). Other than R^2, V_{max} (9.6419), V_{max}/K_s (0.0606) and K_s (159.0271) are also very effective tools in estimating biodegradation efficiency of isolate. An enzyme having lower K_s value is generally saturated and works with steady rate. Such enzymes efficiency is not significantly altered with minor changes in substrate concentration.

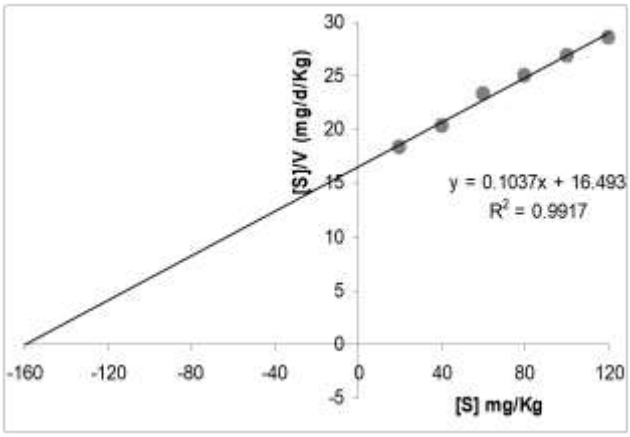

Figure 6. Hanes plot for biodegradation kinetics.

***Conclusion*:** Excessive use of pesticides are negatively effecting microbial communities in agricultural soils and resulting in fertility loss. Presently, most eco-friendly solution is bioremediation. Present study is successful as we were able to isolated CP resistant *Pseudomonas* sp from industrial drain. This strain effectively degraded CP and its metabolites in laboratory soils with any toxic metabolite formation. With these initial results, we hope that this strain can be used to restore CP contaminated soils, but field trials were not the part of this research project.

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