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Enhanced Biodegradation of Chlorpyrifos by Agricultural Soil Isolate

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Pesticide pollution is increasing day by day and most of them are persistent in environment. In the present study, we isolated microbial strains from cotton growing agricultural soils which are under widespread use of chlorpyrifos (CP). The chlorpyrifos tolerance limit of isolated strains differs significantly from one another. Ct3 (*Bacillus cereus*) was most resistant and efficient in degrading chlorpyrifos. Number of parameters that affect rate of biodegradation and efficiency were investigated. These parameters include, chlorpyrifos concentration, alternate carbon source, inoculum size and pH. Stain Ct3 was able to utilize chlorpyrifos as carbon and energy source and also show efficient degradation in presence of glucose as alternate carbon source. Enhanced biodegradation in presence of glucose may be due to the rapid increase in number of bacteria. Ct3 was more efficient at pH 8.5 and high inoculum density. However, the recommended inoculum size is 10^6 cells L^{-1}. Maximum degradation achieved was 88 %, with the initial concentration of 300 mg L^{-1}. The time taken for this was 7 days. This study was successful in chlorpyrifos biodegradation and this can be used for ecological restoration of sites contaminated with chlorpyrifos.

Keywords: Bioaugmentation, Biodegradation, Chlorpyrifos, Eco-restoration, Kinetics.

INTRODUCTION

Now days, pesticides are the important component in agriculture. Pesticide use is increasing, due to increasing pest attack and continuously increasing population. It is believed that world's food demand cannot be achieved without use of pesticides. These pesticides are detrimental for ecosystem health and food chain[1]. Most of the pesticides are resistant towards degradation and remain available in soil/water for long durations. The problem in further aggravated by the use of those pesticides which has high bioaccumulation potential. These pesticides enter in food chain *via* different routes and then accumulate in organisms. Their concentration increases from one trophic level to next trophic level[2]. Among different types of pesticides, organochlorine and organophosphates are more widely used. Organochlorine is more bioaccumulative, target non-pest organism and are persistent. Because of these problems organophosphates are replacing organochlorines. Organophosphates have more efficacies and less persistence but are neurotoxic[3].

Chlorpyrifos (CP) is widely used organophosphate insecticide against mosquitoes, flies, termites and number of soil insects. Its degradation and half life depend on concentration, bioavailability, presence of nutrients, substrate, prevailing environmental conditions, pH, growth rate and inoculum size[4]. It may persist for more than one year. Earlier researchers were on the view that alkaline nature of soil is responsible in pesticide dissipation from the environment. The mechanism hypothesized was of hydrolysis[5]. Further investigations revealed that pesticides dissipation in not possible in sterile soils. These and similar results highlighted the importance of soil micro flora and fauna in pesticide removal from the soil. The process involved in this removal is biodegradation. This initiated the attempt to isolate more resistant and potent microorganism that could degrade chlorpyrifos[6].

As a rule of thumb, those soils which are under extensive and frequent pesticide spray contain more resistant bacteria. Over the years these bacterial strains may have evolved characteristic features that facilitate them to withstand the toxicity of pesticide and degradation[7]. Such microbes are the possible solution of soils contaminated with pesticides and may be used for eco-restoration projects. One of the biggest hindrance in finding chlorpyrifos degrading microbe is that one of its metabolite (3,4,6-trichloro-2-pyridinol, TCP) is also toxic. For that reason, to degrade chlorpyrifos, microbe must be tolerant against both, chlorpyrifos and 3,4,6-trichloro-2-pyridinol[8].

Singh *et al.*[9] isolated *Enterobacter sp*. from soil contaminated with chlorpyrifos. This strain was competent for superior biodegradation. Similarly, Li *et al.*[10] successfully isolated *Sphingomonas sp*. which can degrade 0.1 g L^{-1} chlorpyrifos into 3,4,6-trichloro-2-pyridinol. On the other hand, *Pseudomonas sp*. was more potent and effective in degrading atrazine at basic pH. Elevated organic content also facilitate this bacteria[6]. In the same way, inoculum size also play vital role. Generally, recommended inoculum size for *in situ* bioremediation is 10^6-10^8 cells g^{-1} of soil. Conversely, 10^5 cells g^{-1} of *Agrobacterium* strain was sufficient for rapid biodegradation[11]. Isolated microbes also need to be optimized against pesticide concentration in order to prevent failure of bioremediation. Chlorpyrifos metabolize into 3,4,6-trichloro-2-pyridinol which has more solubility in water compared to chlorpyrifos. Fate of 3,4,6-trichloro-2-pyridinol in environment is not well understood[2].

Present study was designed to investigate the agricultural soil for isolation of chlorpyrifos resistant bacteria, which can degrade chlorpyrifos. Secondly, behavior of isolates at high chlorpyrifos concentration will be investigated. Thirdly, to achieve the maximum and rapid degradation, important factors were also optimized.

EXPERIMENTAL

Soil sampling: Areas from where soil samples were collected include, Rajan pur, Multan, Faisalabad, Muzafaraghar, Bahawalpur and D.G. Khan. Protocol used for sampling was of Tariq *et al.*[3].

Isolation and identification of microbial strains: For isolation, approximate 30 g of soil was mixed with sterile minimal salt medium (MSM) in a flask. Later 25 mg L^{-1} of chlorpyrifos was added in flask and was shaked at the speed of 100 rpm. After 1 week, 10 mL culture was transferred to fresh minimal salt medium containing pesticide. Same procedure of transferring culture to fresh minimal salt medium was repeated after every week. Increased concentration of pesticide is used in every successive week. For screening and isolation maximum concentration of chlorpyrifos used was 150 mg L^{-1}. Loss in minimal salt medium volume due to evaporation was maintained by adding appropriate volume of distilled water. From the last sub-culture, 10 fold serial dilutions were prepared and subsequently each dilution (150 µL) was spread on nutrient agar plate. These nutrient agar plates also contain 150 mL L^{-1} of chlorpyrifos. That bacterial strain which is resistant against chlorpyrifos showed growth on nutrient agar plates. Resistant colonies were picked and further purified to get monoculture[12]. Morphological, physiological and biochemical characteristics of selected isolate was used for its identification using Bergey's Manual of Determinative Bacteriology[13].

Inoculum preparation: Seed culture was prepared in nutrient broth. Centrifugation of seed culture was carried at 4600 rpm for approximately 10 min. Pellets were washed with sterile N-saline (0.9 %) and were resuspended again in 0.9 % N-saline. Optical density was measured at the wavelength of 590 nm and seed culture quantification was done by dilution plate count method[14].

Biodegradation of pesticide: Shake flask study was conducted to investigate the chlorpyrifos biodegradation potential of selected isolate. 30 mL minimal salt medium, pesticide (known concentration) and inoculum (quantified) was mixed in a flask[15]. Constant incubation (37 °C) and shaking (100 rpm) was carried for up to 12 days. For the purpose of compassion, control was also set without inoculum. All the flasks were set in triplicates. To measure the biodegradation rate and quantity, sample were drawn (aseptically) from flasks[14]. The present research only deals with the degradation of chlorpyrifos not its metabolites.

To study the effect of chlorpyrifos concentration on rate of degradation and microbial behavior 3 concentrations were used *i.e*. 200, 300 and 400 mg L^{-1}. In each experimental setup only chlorpyrifos concentration was changed and rest of the condition remained same. Carbon sources tested were, glucose, mannose and starch. Similarly, degradation pattern at different inoculum size was also investigated by changing inoculum size from 10^4-10^8 CFU mL^{-1}. pH was optimized by conduction experimentations in acidic, neutral and basic pH.

Kinetics study: Michaelis-Menten model was used to calculate biodegradation kinetic constants[16]. 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, K_s = half saturation constant

Extraction and HPLC analysis of chlorpyrifos: 10 mL sample was centrifuged (7200 rpm) for 10 min to separate bacterial cells from the liquid medium. In supernatant, same volume of dichloromethane was added, shaked and organic layer was collected. Later dichloromethane was evaporated at room temperature and filtered to remove any particle. Residues were dissolved in acetonitrile and filtered again, in order to extract the entire chlorpyrifos. 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; 20 µL sample volume, 1 mL min^{-1} flow rate, 15 min retention time and 290 nm wavelength[12].

Statistical analysis: Statistical analysis was done through SPSS and Costat software.

RESULTS AND DISCUSSION

Eco-friendly solutions for pesticide contamination are the need of an hour. Biodegradation by microbes is considered one of the potential options for *in situ* restoration of contaminated sites. *In situ* restoration is effected by many factors like, chemical nature of pesticide, pesticide toxicity, affinity with soil, pH, nutrients availability, moisture and temperature[17]. Both success and failure of pesticide biodegradation is reported. For instance, pentachlorophenol was successfully degraded up to 80 % by using *Sphingobium chlorophenolicum* with an initial concentration of 100 mg kg^{-1}. This strain was also very potent when inoculated in soil[18]. Similarly, Niu *et al.*[19] investigated accelerated metabolism of 4-chloronitroben-zene in soils inoculated with mono culture of *Pseudomonas putida* ZWL73, while sterile soil does not showed enhanced degradation.

Burkholderia sp. strain FDS-1 completely metabolizes 15 mg kg^{-1} of fenitrothion in just 15 days. Whereas, up to 30 % metabolization was observed in control[20]. The utility of bioaugmentation in extreme environmental conditions is also proved. For instance, 75 % of oil degradation in Antarctic soil was carried out by psychrotolerant strains whereas, autochthonous communities were less efficient and degradaed only up to 35 %. These psychrotolerant include *Acinetobacter sp.* and *Acinetobacter johnsonii*[21]. Beside bacteria, fungi could also be the strong candidate for bioremediation. 99 % fluorine was degradaed by *Absidia cylindrospora* in 12 days, while same degradation in in-inoculated soil was achieved in 24 days[22]. Complete mineralization or biodegradation involves oxidation of parent molecules and as a result water and carbon dioxide are produced. This carbon and energy source is used for growth and reproduction by microbes. Being complex in nature, each step of biodegradation is catalyzed by specific enzyme. Absence of specific enzyme is one of the leading reasons of failure in pesticide degradation. Bioaugmentation is the recommended option if specific microbes are not available for biodegradation or if their number is much low[23,15].

Isolation and characterization of chlorpyrifos degrading bacterium: In the present study, twenty four strains were isolated from agricultural soil, capable of degrading chlorpyrifos. Ct3, Ct6 and Ct10 were more resistant among 24 isolates and showed good growth (Table-1). Other strains are variably sensitive. For detailed investigation only Ct3 was used. This isolated strain was able to use chlorpyrifos as carbon and energy source. By using this carbon and energy these microbes grow and increase their number[23].

TABLE-1
SCREENING OF ISOLATES

Isolates	Chlorpyrifos concentration (mg L^{-1})				
	75	100	125	150	175
Ct 3	+++	+++	+++	+++	+++
Ct 6	+++	+++	+++	++	++
Ct 8	++	++	+	–	–
Ct 10	+++	+++	+++	++	++
Ct 11	++	++	+	–	–
Ct 18	+++	++	+	–	–

+++ = Very good growth, ++ = Moderate growth, + = Poor growth, – = No growth

Identification of strain Ct3: On nutrient agar plates Ct3 strain appeared as small and circular colonies. Colonies elevation is convex with entire margins. It exhibit Gram-positive reaction with rod cell shape and are motile. Based on the characteristics and protocol given in Bergey's Manual of Determinative Bacteriology[13], Ct3 was identified as *Bacillus cereus*.

Effect of chlorpyrifos concentration: From the Fig. 1, it can be stated that in the start the biodegradation is slow. Up till 48 h only 10 % degradation was observed. But subsequent to 48 h speedy biodegradation of chlorpyrifos was observed. These results are highly significant as compared to the control which shows minimal degradation. The early 48 h represent the lag phase of bacterial growth in which microbes are not much adaptive to the changed environment and secondly their number is low. This low microbe number cannot lead to the rapid degradation. Whereas, time after 48 h represent log phase where the bacterial growth rate and degradation rate is maximum. From the results it can be concluded that the degradation percentage is inversely proportional to the chlorpyrifos concentration. More the concentration less is the degradation. Maximum degradation reported was 67, 80 and 88 % with initial concentrations of 400, 300 and 200 mgL^{-1}, respectively. High concentrations do not have inhibitory effect every time, as Struthers *et al.*[11] achieved degradation of high concentration of ethoprophos. Singh *et al.*[9] was able to use maximum of 250 mg L^{-1} of chlorpyrifos for biodegradation. They reported longer lag phases at higher chlorpyrifos concentration. According to Karpouzas and Walker[24], the possible explanation of these longer lag phases is the need of bulky microbial number.

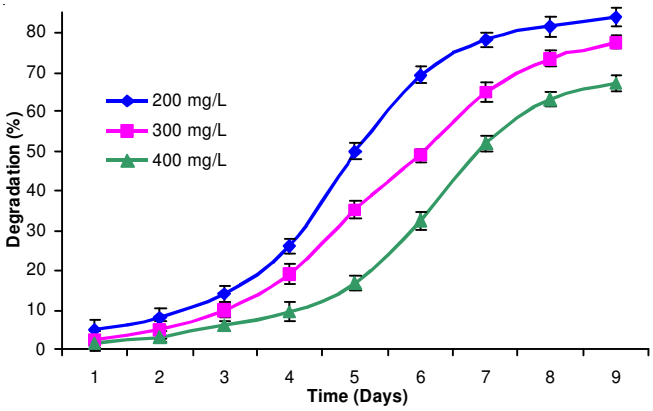

Fig. 1. Effect of chlorpyrifos concentrations on its biodegradation

Effect of media pH: Change in pH leads to the change in chemical nature of xenobiotic compounds and thus significantly influence the biodegradation. *B. cereus* exhibited chlorpyrifos degradation in both acidic and basic pH. Acidic pH was more suitable for *B. cereus* and more degradation is observed at acidic pH (Fig. 2). Somewhat similar trend with the change in pH was reported by Singh[6], rapid and more degradation was observed by him at higher pH by *Enterobacter sp.* on the contrary, pH 7.6-5.5 is more favorable to support rapid degradation of ethoprophos (organophosphate) pesticide[24]. In the study, maximum degradation was observed at higher pH. This may be due to the optimum pH requirement of enzymes involved in chlorpyrifos degradation. Present study validate the use of *B. cereus* for eco-restoration projects which will lead to the cleaner environment and economic growth.

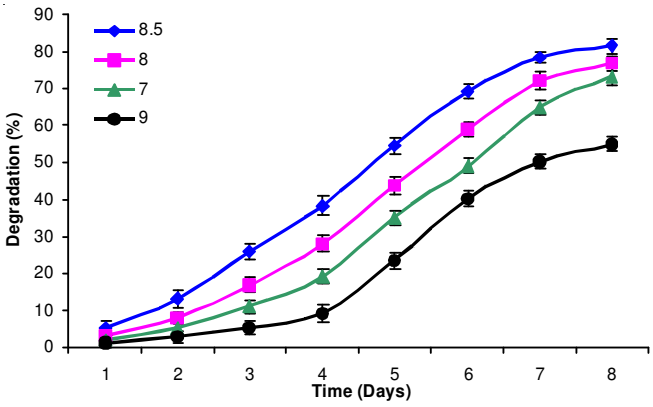

Fig. 2. Effect of pH on chlorpyrifos biodegradation

Effect of carbon source: Presence and absence of carbon source may lead to the change in biodegradation pattern by microbes. In the presence of easily degradable carbon source, microbe will not target pesticides thus leads to the failure of bioremediation. In present study, 3 carbon sources were tested. In presence of glucose, yeast extract and starch the maximum degradation was up to 87, 78 and 70 %, respectively in 7 days (Fig. 3). Results showed that the glucose is the most efficient carbon source in enhancing degradation of chlorpyrifos. In 6 days, nearly 80 % chlorpyrifos was degraded. Singh *et al.*[9] reported somewhat contradictory results, according to him *Enterobacter* strain do not degrade chlorpyrifos at start (in presence of glucose) but after 36 h the degradation process starts again. These results validate the potential of *B. cereus* to degrade chlorpyrifos in presence to nutrient rich medium.

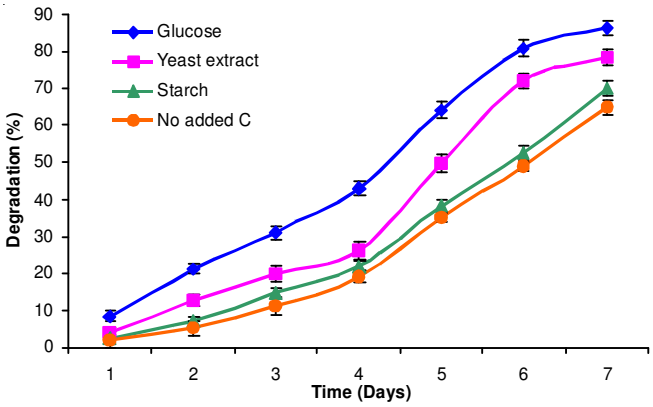

Fig. 3. Effect of different carbon sources on chlorpyrifos biodegradation

Effect of inoculum density on degradation of chlorpyrifos: For efficient and rapid degradation minimum number of microbes is required. Number below it leads to slow degradation and number above it leads to the death of microbes. In present study different range of inoculum densities were tested which ranged from 10^4 to 10^8 CFU mL^{-1}. Fig. 4 showed that the inoculum size has proportionate relation with degradation percentage. Degradation at all the inoculum sizes is significantly different from one another. With 10^8 CFU mL^{-1} 90 % degradation was achieved in 4 days, having very short lag phase. Alternatively, low inoculum size leads to 60 % (10^6 CFU mL^{-1}) and 19 % (10^4 CFU mL^{-1}) in 4 days. Generally, before rapid degradation longer lag phases appeared. Chen *et al.*[25] suggested that this longer lag phase is the time required by the microbe to increase their number for biodegradation. Low microbial number cannot lead to rapid degradation.

Kinetic study of biodegradation: Apparently, enzyme having high valve of K_s has low substrate affinity and subsequently it need high substrate concentration to attain V_{max}. 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

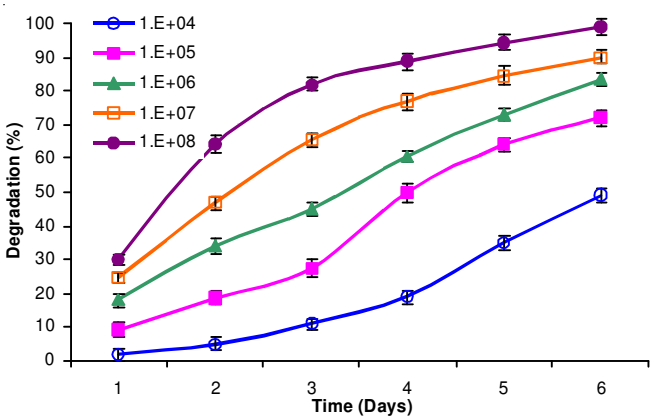

Fig. 4. Effect of inoculum density on chlorpyrifos biodegradation

at different substrate concentration. The result will give hyperbolic curve, when substrate concentration [S] is plotted against reaction rate [V][16]. Biodegradation kinetics of *Bacillus sp.* and *Pseudomonas sp.* was measured in the range of 100-500 mg L^{-1} of chlorpyrifos. Fig. 5 represents the biodegradation kinetics of chlorpyrifos by *Bacillus sp.* ($R^2 = 0.9983$). The high value of R^2 represents the best fit of data into Michaelis-Menten equation. *Bacillus sp.* is considered the best for biodegradation processes.

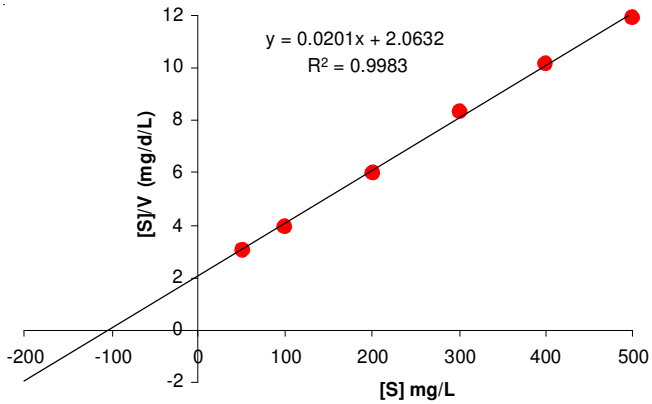

Fig. 5. Hanes plot, for calculating chlorpyrifos biodegradation kinetics

Conclusion

Biodegradation is gaining popularity as eco-friendly approach for ecological restoration. One of the key steps for successful bioremediation is to select microbial strain, which have a potential to degrade specific pollutant. Those sites which are under massive and continuous exposure of pesticide are excellent source of microbes for bioremediation. Indigenous microbes which can survive with other microflora and fauna are better option than others. Abiotic factors also need to be optimized for successful bioremediation. In conclusion, present results validate the use of isolated chlorpyrifos degrading bacteria for bioremediation of contaminated sites.

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