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Research Article

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Optimization of Chlorpyrifos degradation by *Pseudomonas putida*

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ABSTRACT

A total of 58 isolates capable of degrading chlorpyrifos were obtained out of 27 samples collected. Among them 10 isolates showed better efficiencies in degrading chlorpyrifos as compared to others. Isolate CHS23a which showed 38% degradation of chlorpyrifos under unoptimized condition was identified as Pseudomonas putida. After optimization process it was able to show 76% degradation of chlorpyrifos at pH 7, temperature 35°C, pesticide concentration of 2%, 10 ml of inoculum size, shaking speed of 150 rpm and in presence of 200 mg/l glucose and 300 mg/l yeast extract.

Keywords: Chlorpyrifos degradation, optimization, *Pseudomonas putida.*

INTRODUCTION

Synthetic organophosphates are widely used to control various pests for agriculture and for public health protection and these account for approximately 38% of total pesticides used globally [1]. Chlorpyrifos [O, O-diethyl *O*-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate] is one of the most commonly and widely used commercial organophosphorous insecticides [2, 3]. It is effective in controlling a variety of insects including cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants and lice. It has been used as an insecticide on grain, cotton, fruit, nut and vegetable crops as well as on lawns and ornamental plants [4]. It is available in emulsifiable concentrate, dust, flowable, pellet, spray, granular and wettable powder formulations [5].

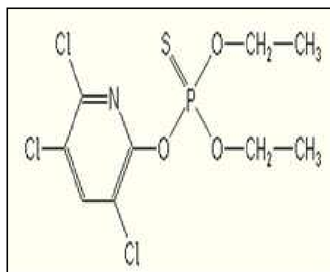
Microbial degradation of organophosphate pesticides is of particular interest because of the high mammalian toxicity of such compounds and their widespread and extensive use [6]. Chlorpyrifos is a neurotoxin and suspected endocrine disruptor and it has been associated with asthma. Recent research indicates that children exposed to chlorpyrifos while in the womb have an increased risk of delays in mental and motor development at age 3 and an increased occurrence of pervasive developmental disorders such as attention-deficit hyperactivity disorder [7]. A study demonstrated a correlation between prenatal chlorpyrifos exposure and lower weight and smaller head circumference at birth [8]. In plants there have been reports of delayed seedling emergence, fruit deformities and abnormal cell division upon prolonged exposure to chlorpyrifos [9]. Extensive use of chlorpyrifos contaminates air, ground water, rivers and lakes. The contamination has been found upto about 24 kilometers away from the point of use [10].

Considerable amount of work has been done on chlorpyrifos degradation by bacteria [11, 12, 13, 14, 15,16] and fungi [17, 18, 19] isolated from agricultural soil and other sources. With the aim of isolating efficient strains of

bacteria capable of degrading chlorpyrifos and to optimize various parameters for degradation of chlorpyrifos the present investigation has been taken up.

EXPERIMENTAL SECTION

Pesticide and other chemicals: Commercial-grade insecticide chlorpyrifos was procured from a pesticide selling shop in Bangalore. Other chemicals were procured from Hi-Media Pvt. Ltd., Mumbai.



Structure of Chlorpyrifos

Isolation, enrichment and screening: Twenty seven soil samples were collected in sterile polythene bags from different sites in and around Bangalore having a history of repeated application of chlorpyrifos. One gram of soil samples were inoculated into 100 ml of mineral salts media [20] enriched with 1% chlorpyrifos as the sole source of carbon. Flasks were incubated for 90 days at 37°C under static condition with intermittent addition of 1% pesticide for enrichment of cultures. Bacteria capable of degrading chlorpyrifos were isolated on mineral salts agar medium with 1% pesticide as sole source of carbon.

Loopfuls of each cultures were inoculated into fresh mineral salts media with chlorpyrifos. These cultures were incubated till log phase. One ml of inoculum from each flask were inoculated into 100 ml of fresh mineral salts medium with pH 7 containing 1% pesticide and the flasks were incubated at 37°C under static condition. To monitor the growth of cultures O.D. was measured at 660 nm every 24 hrs. Cultures showing higher O.D. by the end of 7 days were selected for further work.

Loopfuls of cultures were also inoculated into mineral salts agar media containing 1% pesticide and 0.02% phenol red indicator [21]. Cultures showing maximum zone of colour change from red to yellow due to production of acidic products were selected for further studies.

Further screening of the isolates was carried out by estimating the amount of chlorpyrifos according to Khan *et al.* [22]. Percent degradation of the pesticide by the cultures was calculated using the formula Percent degradation = [(Initial absorbence-Observed absorbence)/Initial absorbence]x100

Identification of Bacteria: Preliminary identifications of all the cultures were done using Gram's staining. The isolate CHS23a which showed better degradation efficiency was identified upto the genus level by biochemical tests [23] and upto species level based on nucleotide sequence and phylogenetic tree analysis. Nucleotide sequence was deposited in NCBI Gene Bank.

Optimization of degradation of chlorpyrifos: Optimization of degradation of chlorpyrifos by *Pseudomonas putida* with respect to various parameters like pH (between 4 to 10), temperature (30°C to 50°C), inoculum concentration (1 to 25 ml), pesticide concentration (2% to 10%), nutrient sources like carbon (Mannose, Starch, Lactose, Sucrose and Glucose) nitrogen (KNO_3, Peptone, $NaNO_3$, NH_4NO_3 and Yeast extract), aeration and agitation (with different shaking speeds 50 to 250 rpm) was carried out. Degradation was carried out with different concentrations of Glucose (100 mg/lit to 500 mg/lit) and Yeast extract (100 mg/lit to 500 mg/lit) added to mineral salts medium containing chlorpyrifos. Degradation efficiency of the culture was obtained by estimating pesticide concentration according to Khan *et al.* [22]. Degradation of pesticide was carried out under optimized conditions and result was compared with that under unoptimized conditions.

RESULTS

Isolation, enrichment and screening: A total of 58 isolates capable of degrading chlorpyrifos were isolated out of 27 samples collected. Among them 23 isolates were able to show good growth in mineral salts medium with chlorpyrifos as sole source of carbon. Majority of the isolates were gram negative in nature. On mineral salts agar media containing pesticide and phenol red, 10 isolates showed prominant zone of colour change from red to yellow due to production of acidic products (Fig. 1). Among them CHS23a was able show 50% degradation of chlorpyrifos.

Fig. 1: Growth of isolates on medium with phenol red

Identification of Bacteria: Based on biochemical tests the isolate CHS23a was tentatively identified as *Pseudomonas* spp. Based on nucleotide sequence and phylogenetic tree analysis its identification was confirmed (Fig. 2 and 3) as *Pseudomonas putida.* Nucleotide sequence of the same was deposited in the Gene Bank with the accession number JQ701740.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP002290.1	Pseudomonas putida BIRD-1, complete genome	2688	1.880e+04	99%	0.0	99%
HQ166061.1	Pseudomonas putida strain FBHYA-B3 16S ribosomal RNA ge	2688	2688	99%	0.0	99%
CP000926.1	Pseudomonas putida GB-1, complete genome	2688	1.879e+04	99%	0.0	99%
EF107048.1	Pseudomonas sp. 4zhy 16S ribosomal RNA gene, partial seq	2688	2688	99%	0.0	99%
AM411058.1	Pseudomonas putida partial 16S rRNA gene, strain 5zhy	2688	2688	99%	0.0	99%
DQ229317.1	Pseudomonas putida isolate BCNU154 16S ribosomal RNA ge	2688	2688	100%	0.0	99%
AE015451.1	Pseudomonas putida KT2440 complete genome	2688	1.878e+04	99%	0.0	99%
EF392658.1	Pseudomonas sp. PD1 16S ribosomal RNA gene, partial sequ	2686	2686	99%	0.0	99%
HM152684.1	Uncultured Pseudomonas sp. clone Filt.97 16S ribosomal RN	2684	2684	99%	0.0	99%
GU828030.1	Pseudomonas putida strain 75 16S ribosomal RNA gene, par	2684	2684	99%	0.0	99%

Fig. 2: Sequence alignment of CHS23a

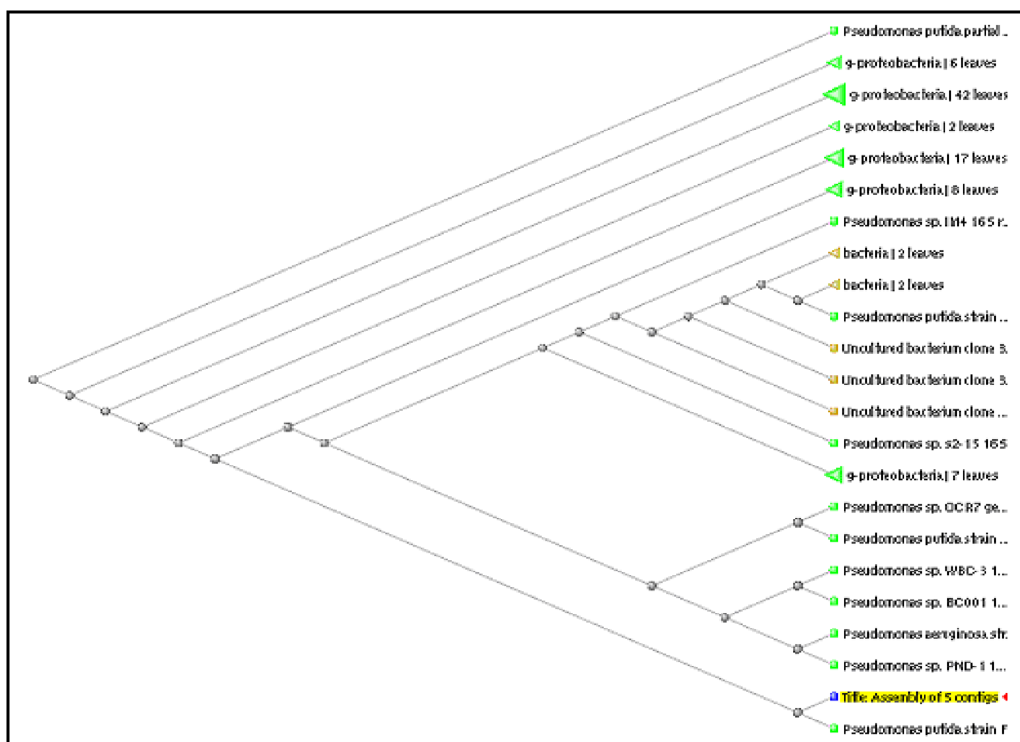


Fig. 3: Phylogenetic tree of CHS23a

Optimization of degradation of chlorpyrifos: When effect of pH was studied on degradation of chlorpyrifos by *Pseudomonas putida* maximum degradation of 38% was seen at pH 7. Least degradation of 24% was seen at pH 5 (Graph 1). *Pseudomonas putida* showed maximum degradation of 36% at 35^0C. Least degradation of 21% was recorded at 50^0C (Graph 2).

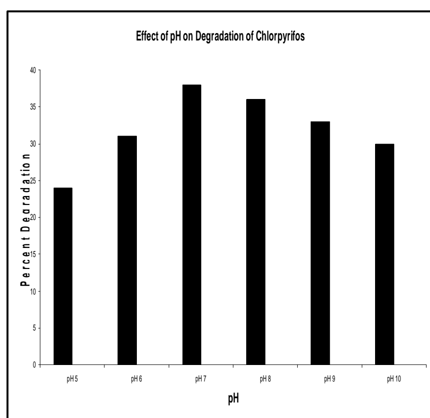
Difference in inoculums size had little effect on degradation efficiency of *Pseudomonas putida*. Maximum degradation of 42% was seen with inoculum size of 10 ml. A least degradation of 38% was seen with inoculum size of 1 ml (Graph 3). Higher concentration of pesticide resulted in decreased efficiency of *pseudomonas putida*. Maximum Degradation of 38% was seen with pesticide concentration of 2%. Least degradation of 20% was seen with pesticide concentration of 10% (Graph 4).

With increase in shaking speed percent degradation of chlorpyrifos by *Pseudomonas putida* also increased upto 150 rpm. Further increase in shaking speed resulted in decreased efficiency of *Pseudomonas putida*. Maximum degradation of 62% was seen with shaking speed of 150 rpm. Least degradation of 18% was seen with shaking speed of 250 rpm (Graph 5).

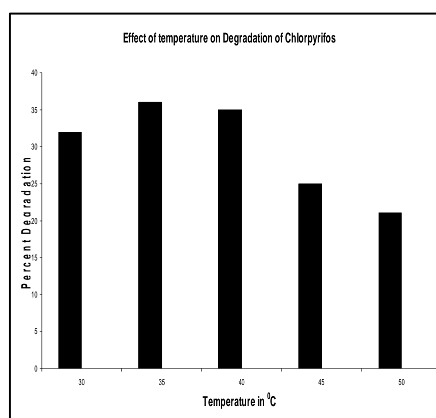
With the addition of carbon source *Pseudomonas putida* showed better degradation of chlorpyrifos as compared to that in absence of any additional carbon source. Maximum degradation of 57% was seen with glucose as the source of carbon. Least degradation of 40% was seen with sucrose as the source of carbon (Graph 6). Further when effect of different concentration of glucose was studied, maximum degradation of 65% was seen with 200 mg/l of glucose. With higher concentration of glucose percent degradation decreased. Least degradation of 42% was seen with 500 mg/l of glucose (Graph 7).

Similar results were obtained with the addition of nitrogen source. Maximum Degradation of 68% was seen with yeast extract as the source of nitrogen. Least degradation of 40% was seen with KNO_3 (Graph 8). When effect of different concentration of yeast extract was studied, maximum degradation of 70% was seen with 300 mg/l of Yeast extract. Least degradation of 59% was seen with 500 mg/l of Yeast extract (Graph 9).

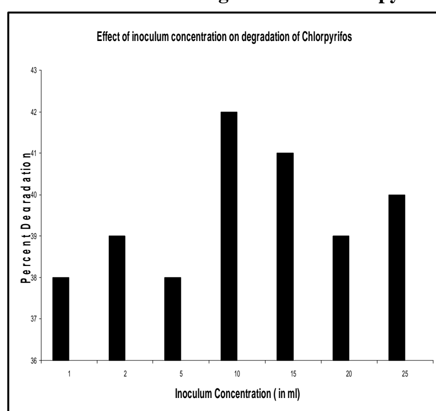
Degradation under optimized condition: Degradation of 76% was recorded at the end of 24 hrs with 2% pesticide concentration, 10 ml inoculum size, 200 mg/l of glucose and 300 mg/l of yeast extract, at 150 rpm shaking speed, at 35^0C and at pH 7 as compared to 38% degradation recorded at the end of 120 hrs under unoptimized condition of pH 7, temperature 35°C, 2% pesticide concentration, 1 ml of inoculum size, under static condition and in absence of additional carbon and nitrogen source (Graph 10 and 11).



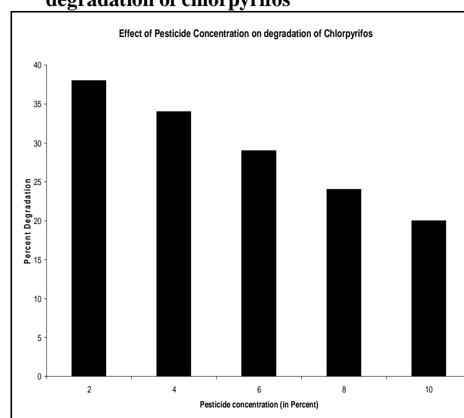
Graph 1: Effect of pH on degradation of chlorpyrifos



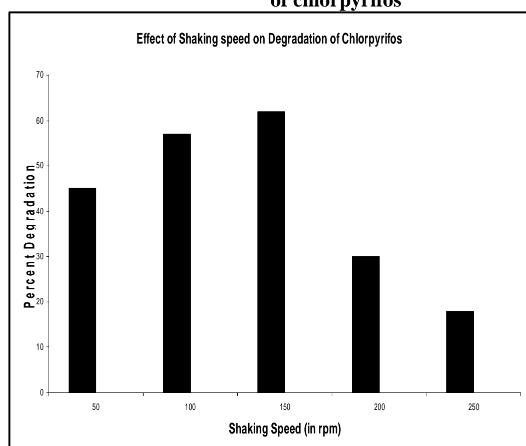
Graph 2: Effect of temperature on degradation of chlorpyrifos



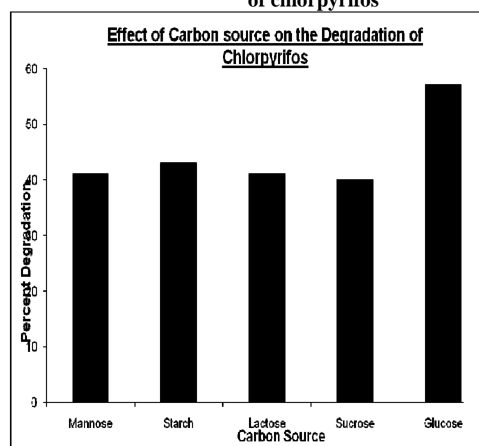
Graph 3: Effect of Inoculum concentration on degradation of chlorpyrifos



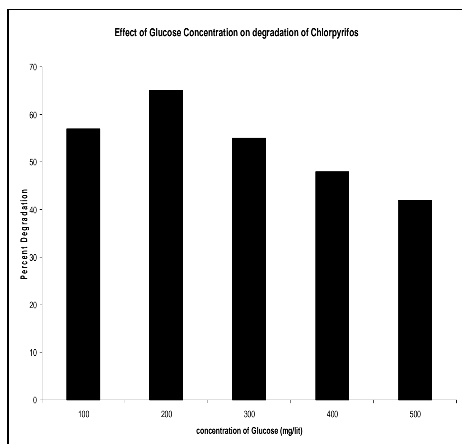
Graph 4: Effect of Pesticide concentration on degradation of chlorpyrifos



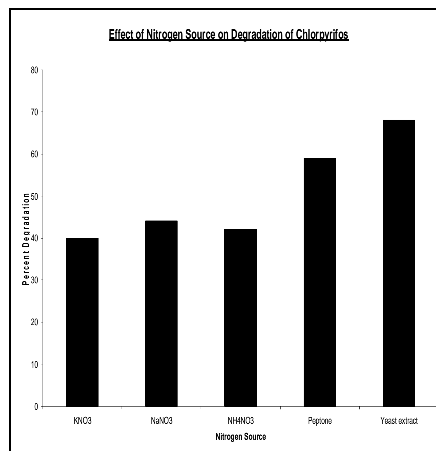
Graph 5: Effect of Shaking speed on degradation of chlorpyrifos



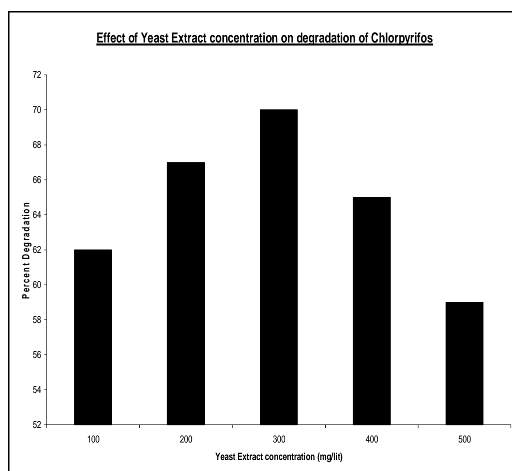
Graph 6: Effect of Carbon source on Degradation of Chlorpyrifos



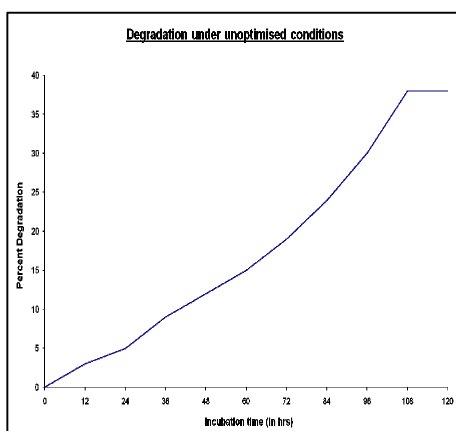
Graph 7: Effect of Glucose concentration on degradation of chlorpyrifos



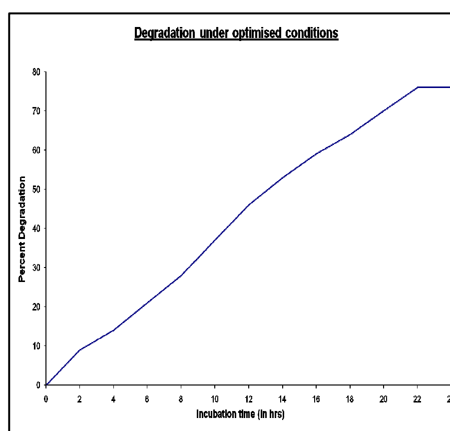
Graph 8: Effect of Nitrogen source on degradation of chlorpyrifos



Graph 9: Effect of Yeast extract concentration on degradation of chlorpyrifos



Graph 10: Degradation under unoptimised conditions



Graph 11: Degradation under optimised conditions

DISCUSSION

Few species of *Pseudomonas* have been used for degradation of chlorpyrifos either alone or as a part of a consortium [24, 25, 26, 27]. Efficient degradation of chlorpyrifos using *Pseudomonas putida* has been carried out by Ajaz *et al*. [28]. In the current study, the isolate CHS23a showing highest degradation was identified as *Pseudomonas putida.*

For screening of bacterial isolates capable of degrading chlorpyrifos, mineral salts medium containing pesticide and phenol red indicator was used in the present study Veeranagouda *et al* [21] have carried out degradation of dimethylformamide using phenol red as an indicator in the media. Use of bromothymol blue in the media to check for change in pH due to pesticide degradation has been demonstrated by George [29]. *Pseudomonas putida* grows best at neutral pH and at mesophilic range of temperature. Optimum degradation of chlorpyrifos by *Pseudomonas putida* was observed at pH 7, temperature 35 °C, pesticide concentration of 2%, 10 ml of inoculum and shaking speed of 150 rpm. Degradation was enhanced in presence of 200 mg/l of glucose and 300 mg/l of yeast extract. Liu *et al* [30] have shown that chlorpyrifos could be degraded by *Bacillus cereus* at 30°C, pH 7 and 100 mg/l of chlorpyrifos concentration. According to Awad *et al.* [31], *Pseudomonas* spp. showed optimum degradation of chlorpyrifos at pH7, temperature 30°C, inoculum size of 0.5 ml, chlorpyrifos concentration of 0.1-0.35 ml/l and shaking speed of 100 rpm. Fructose was preferred as a carbon source and ammonium nitrate as a nitrogen source by *Pseudomonas* spp.

Decrease in degradation efficiency of *Pseudomonas putida* was observed at higher speed of shaking condition. This could be due to less contact between the pesticide and the culture. Similar results in higher concentration of glucose and yeast extract could be due to preferential utilization of the same as compared to that of the pesticide. Awad *et al.* [31] have shown that below and above optimal incubation condition of pesticide concentration, inoculum size, temperature, pH and additional carbon and nitrogen source degradation efficiency of *Pseudomonas* spp. decreased. Under unoptimized condition *Pseudomonas putida* showed highest degradation in 10 days whereas under optimized condition culture was able to show better efficiency within 24 hrs.

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