BIODEGRADATION OF CHLOROPYRIFOS BY Actinomycetes SPECIES IN AQUEOUS PHASE

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Received September 14, 2013 Accepted February 15, 2014

ABSTRACT

Currently, the pollution potential of many surface and ground waters with respect to chloropyrifos (CPF) and other toxic pesticide residues is significantly alarming and many physico-chemical treatment techniques are infeasible and/or highly uneconomical. Liquid phase biodegradation of CPF by a suitable and appropriate organism is necessary. Prior to biodegradation studies, the Actinomycetes sp. was extracted, isolated and identified through appropriate Gram staining and biochemical test (including selective media for growth of Actinomycetes sp.). The biodegradation of CPF was almost 100% at the low concentration levels between 0.5 and 2 mg/l on the other hand, for higher concentration of CPF, incomplete biodegradation could be noticed. Approximately 98.3% (around a neutral pH of 7) and beyond pH 8, complete removal could be achieved. The biodegradation kinetics was appropriately described by simple first-order kinetics with the rate constant almost doubled (between 0.04 and 0.08 1/h respective CPF concentrations of 2 and 5 mg/l). For the concentrations of CPF between 2 and 5 mg/l, the yield coefficient varied between 0.92 and 0.13 g/g, respectively, specific growth rate varied between 0.84 1/h and an average endogenous decay coefficient 0.016 1/h. In all the biodegradation studies pertaining to monoculture and/or mixed culture bacteria, lindane toxicity pressure was significantly noticed even after 24 h.

Key Words: Chloropyrifos, Actinomycetes, Biodegradation, Lindane, Biochemical test, Monoculture, Mixed culture

INTRODUCTION

The agricultural development and industrial growth are the two integral components of sustainability of India, after its independence. The technical grade manufacture of DDT (first synthetically prepared chemical: organochlorine insecticide), more than 3,000 broad-band insecticides (including CPF) are being produced throughout the world. The production and application of technical grade CPF and/or its formulations in India is ever increasing from decade to decade, in spite of other organophosphatic pesticides like parathion, malathion and others. Although the production of CPF in Tamilnadu is relatively small, when compared to its neighboring states like Karnataka, Kerala and Andhra Pradesh but substantial amount of CPF is being applied both in agricultural and nonagricultural sectors. Even though the chief source of CPF pollution is due to manufacturing and/or formulation activity, its widespread presence in all the components of environment is through diversified direct and/or indirect applications. Owing to its vital neurotoxic effect in human beings, its presence (either as CPF or as its oxon form in surface and/or ground waters should be well below 0.01 µg/l (as per several international bodies and IS: 10500-1995). To achieve this, many physico-chemical and/or biological treatments have been adopted onsite so as to completely eliminate its residues, before the treated effluent reaches the receiving water body. But, their overall cost was quoted to be substantially higher due to the incorporation by multiple diversified units like neutralization, coagulation, sedimentation, chlorination, filtration, carbon adsorption and others. Although biological treatment was proved to be successful in eliminating CPF residues (at substantially higher concentration i.e., at 100s of mg/l), very scanty information is available about the biodegradation of its residues at few mg/l and/or < 1 mg/l.

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MATERIAL AND METHODS

Extraction of microorganisms from soil
The extraction of appropriate CPF degrading microorganisms was carried out by taking 1 gm of soil from turmeric cultivation field (which was pre-treated with CPF residue), Anthiyur Town, Erode District, Tamil Nadu, India. Later, it was mixed with 100 ml of distilled water and subsequently subjected to serial dilution. From each serially diluted sample, 1 ml of aliquot was spread by L-rodd on the respective standard size petridishes containing nutrient agar. Further, all the petridishes inoculated with microorganisms were kept for incubation at 37º C for 24 h and the respective colony counts were physically counted by digital bacteriological colony counter.

Selection and identification of appropriate culture
The appropriate culture was selected by taking 2 mg/l of initial CPF in respective nutrient broth containing 100 µl of each culture. After 3 days of incubation at 37º C the CPF residue remaining was analyzed.

The gram staining procedure was followed as per the standard procedure. All the biochemical tests (for the biodegradation studies explained later) were performed in Rnd Bio Solutions and Pest Management for the identification of the appropriate bacteria obtained from the gram staining technique. The various biochemical tests adopted were indole, methyl red, Voges-Proskauer, carbohydrate fermentation, citrate utilization, triple sugar iron, gelatine, urease, starch hydrolysis, casein hydrolysis, mannitol salt agar, MacConkey agar, endo agar, catalase and coagulase tests.

Analysis of chloropyrifos
Although chloropyrifos and its residues are accurately and reliably analysed by either Gas Chromatography (GC) or High Performance Liquid Chromatography (HPLC) techniques, majority of the chloropyrifos analysis was carried out by spectrophotometric method UV-Vis spectrophotometer (Systonics 117, India) at 267 nm with substantial accuracy. However, GC method of analysis was also used wherever necessary along with spectrophotometric method.

Effect of initial concentration of CPF
In order to understand the effect of initial concentration of CPF on its biodegradation, various initial concentrations such as 0.5, 1, 1.5, 2, 3, 4, 5 and 6 mg/l of CPF were considered. For each initial concentration of CPF, 100 µl of Actinomycetes sp. was added and further incubated for 3 days, the CPF residues were analysed in each sample.

Effect of inoculum size on CPF degradation
For the two different initial concentrations of CPF (2 and 5 mg/l), separate experiments were performed by adding 10, 50, 100, 150, 200, 250, 300 µl of Actinomycetes sp. rich inocula to each 50 ml of nutrient broth, to understand its effect on the biodegradation of CPF. At the end of 3 days of incubation, the residual CPF concentrations were determined.

Effect of pH on biodegradation of CPF
The effect of pH on CPF biodegradation was performed in unbuffered systems by adjusting the pH by adding either 0.1N NaOH or 0.1N HCl. At a concentration of 5 mg/l of CPF with an Actinomycetes sp. enriched inoculum dose of 150 µl, experiments were performed in pH 4, 5, 6, 7, 8, 9 and 10 media. At the end of 3 days of incubation, the residual CPF concentrations were determined.

Biodegradation kinetics of CPF by Actinomycetes sp.
Separate degradation kinetics experiments were conducted for the two initial concentrations of CPF (2 and 5 mg/l) by using optimum dose of 150 µl of enriched Actinomycetes sp., under an optimum unbuffered pH 7 medium. The respective CPF residues were analysed periodically between 0 and 96h. However, in this experiment also, respective control samples were adopted for proper assessment of CPF residues.

Growth kinetics of Actinomycetes sp.
At four levels of CPF concentrations (1, 2, 5, and 8 mg/l) Actinomycetes sp., growth kinetics experiments were separately performed, both under controlled and CPF-laden nutrient broth media utilising 150 µl of inoculum volume and pH 7. At periodical time intervals, the analytes were analysed for turbidity at 600 nm and CPF residues.

Biodegradation of CPF with lindane by Actinomycetes sp. and other bacteria
The co-degradation of CPF with lindane, both at 5mg/l levels, under pure monoculture and mixed culture of bacteria (containing Actinomycetes sp.,
**Results and discussion**

**Extraction and isolation of microorganisms**

Six types of cultures (C₁, C₂, C₃, C₄, C₅ and C₆) were selected from the turmeric cultivated field each subculture was subjected to trial CPF biodegradation in liquid media. The six cultures isolated from above steps exhibited the specific morphological characteristics and approximate counts.

**Selection and identification of appropriate culture**

C₂ (having approximate count of 90 compared to other cultures) was considered in rest of the experiments. The gram staining result of culture C₂ indicated that the grown up media revealed monoculture, rod-shaped and gram positive bacteria. Table 1 shows the results of biochemical tests performed on the rod shaped gram positive bacteria.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Indole</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>Methylred</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Voges-Proskauer (VP)</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>Glucose</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Sucrose</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>Dextrose</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>Lactose</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Citrate utilization</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>Triple sugar iron</td>
<td>Acid/Alkaline</td>
</tr>
<tr>
<td>10</td>
<td>Gelatin hydrolysis</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>Urease</td>
<td>Positive</td>
</tr>
<tr>
<td>12</td>
<td>Nitrate reduction</td>
<td>Positive</td>
</tr>
<tr>
<td>13</td>
<td>Starch hydrolysis</td>
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<td>14</td>
<td>Casein hydrolysis</td>
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<tr>
<td>15</td>
<td>Mannitol salt agar</td>
<td>Positive</td>
</tr>
<tr>
<td>16</td>
<td>MacConKey agar</td>
<td>Negative</td>
</tr>
<tr>
<td>17</td>
<td>Endo agar</td>
<td>Negative</td>
</tr>
<tr>
<td>18</td>
<td>SCNA</td>
<td>Positive</td>
</tr>
<tr>
<td>19</td>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>20</td>
<td>Coagulase</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Table 1: Biochemical test results on rod-shaped gram positive bacteria

It is concluded that rod–shaped gram positive bacteria belongs to the genera Actinomycetes sp. As of now (from the bulk literature) and is first time utilised as novel bacterium for the biodegradation of CPF in this study.²

**Effect of initial chloropyrifos concentration**

Fig. 1 shows biodegradation of CPF was almost 100% at the low concentrations levels between 0.5 and 2 mg/l. On the other hand, for higher concentration of CPF between 3 and 6 mg/l, incomplete biodegradation could be noticed (96% removal for 3 mg/l and 73.3% removal for 6 mg/l). The incomplete degradation of CPF for the concentrations higher than 3 mg/l was attributed due to its poor solubility (about 1.5 mg/l in nutrient rich medium) in aqueous phase and faster uptake by Actinomycetes sp. However, this may be also due to insufficient time required to completely take-up the CPF residues in the precipitated form.
In order to satisfy the objectives of this study, a very low concentration of CPF (2 – 5 mg/l) was used in further biodegradation studies.

**Effect of density of Actinomycetes sp. on CPF biodegradation**

For an initial concentration of 5 mg/l of CPF, the effect was predominant whereas for 2 mg/l of CPF, it was significant. However, complete degradation of CPF was noticed beyond a density of $2.1 \times 10^5$ CFU/ml for both 5 and 2 mg/l of CPF and hence an optimum density of $2.1 \times 10^5$ CFU/ml was adopted in rest of the experiments. Also, similar behaviour could be noticed in cultures with lower inoculum densities ($10^5$ and $10^7$ CFU/ml) of *B. pumillus*. In general a smaller inoculum density would result in longer lag phase before rapid degradation of CPF started.

**Effect of pH on biodegradation of CPF**

The degradation of CPF by *Actinomycetes* sp. under various pH media, is shown in Fig. 2. *Actinomycetes* sp. degraded about 19.2% of initial CPF (at an acidic pH of 4), approximately 98.3% (around a neutral pH of 7) and beyond pH 8, complete removal could be noticed (Fig. 2). In addition to the biodegradation of CPF by *Actinomycetes* sp. there are other mechanisms such as hydrolysis and volatilisation which are operative in eliminating CPF residues from aqueous media.

**Biodegradation kinetics of CPF**

Fig. 3 reveals the biodegradation kinetics of CPF by *Actinomycetes* sp. both for 5 and 2 mg/l of CPF. Within 24 h, more than 65% of initial CPF was degraded by *Actinomycetes* sp. and beyond 36 hrs (1.5 days) complete degradation (approximately 96% for 5 mg/l) could be seen. Therefore, from this study also, the precipitated
form of CPF would be consumed rapidly by Actinomycetes sp. As per\textsuperscript{10}, the degradation of CPF by the isolated Klebsiella sp. increased to reach 53, 73 and 92%, respectively at the end of 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} d. Also, Bacillus cereus could effectively decrease the CPF residues from 100 mg/l to 26.1 mg/l in 7 d.\textsuperscript{11} Further, a particular strain of Bacillus genera (Bacillus pumillius C2A1) in minimal salt medium could use CPF as a sole source of carbon and energy while degrading it from 100 mg/l to 30 mg/l, 200 mg/l to 40 mg/l and 300 mg/l to 30 mg/l, at the end of 10 d.\textsuperscript{12} However, in all the above studies, high concentrations of CPF adopted.

In order to assess the biodegradation rate by Actinomycetes sp., the data of Fig. 3 were used to fit into the three fundamental rate kinetics (linearised form of zero, first and second–order kinetics). The biodegradation kinetics appeared to follow zero/first/second order kinetics for 2 mg/l of initial CPF whereas, for 5 mg/l of CPF, the validity of second–order kinetics would be increased due to relatively lower correlation coefficient (0.811) and a negative intercept of -0.27. Further, high correlation coefficients were seen for both the concentrations of CPF, among the first- and zero–order kinetics. However, since most of biodegradation kinetics follow first-order kinetics\textsuperscript{13-15}, it can be fairly concluded that the biodegradation kinetics follow first-order kinetics with the corresponding rate constant almost doubled (varied between 0.04 and 0.08 1/h for respective concentrations of CPF between 2 and 5 mg/l).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Biodegradation kinetics of CPF by Actinomycetes sp.}
\end{figure}

Growth kinetics of Actinomycetes sp.

Fig. 4 shows the typical growth curves up to 42 h for CPF concentrations, such as 0, 1, 2, 5, and 8 mg/l. Growth pattern is same for all the CPF concentrations with a very steep exponential phase after 6 h of incubation. Also, an initial lag of 6 h was noticed in all the growth curves related to CPF residues. However, a long stationary phase of about 18 h was noticed in CPF-based growth curves. Although, Actinomycetes sp. could not grow well up to 24 h of lag phase in CPF-free medium, steep growth occurred beyond 6 h of incubation time, in all the CPF-based media. This confirms that the Actinomycetes sp. can take CPF as chief carbon source.

\(\mu_{\text{max}}\) is around 0.84 1/h and the value of \(K_s\) is approximately 0.3 mg/l (with respect to 50\% of \(\mu_{\text{max}}\) value). The Actinomycetes sp. exhibited the specific growth rates varying between 0.09 and 0.54 1/h for 1 and 8 mg/l concentrations of CPF, respectively with an average endogenous decay coefficient of 0.016 1/h was also seen.

\textbf{Biodegradation of CPF with lindane}

Lindane is one such widely being used organochlorine pesticide together with CPF in many agricultural fields, the probable occurrence of these two pesticide residues can be expected. So, in view of above and practical feasibility of treatment of CPF-laden wastewater and/or CPF contaminated water bodies, this study was undertaken. The influence of lindane residue on biodegradation of CPF by Actinomycetes sp. is shown in Fig. 5. It is observed from Fig. 4 that the Actinomycetes sp. could not take up CPF residues due to the toxicity pressure by lindane at 5 mg/l. However, 20\% removal of CPF was observed under the presence of lindane.
Biodegradation of lindane and CPF by mixed culture of bacteria
The co-metabolism of CPF and lindane by the three organisms, such as *Actinomycetes* sp., *Pseudomonas* sp. and *E.coli* is shown in Fig. 6. The presence of mixed culture of bacteria on biodegradation of CPF with lindane slightly enhanced their uptake (Fig. 6) when compared to the *Actinomycetes* sp. alone. The lindane toxicity pressure was slightly reduced due to its uptake (about 28% in 48 h) combinedly and/or individually by the mixed culture of bacteria.
CONCLUSION

Actinomycetes sp. is an effective bacteria in degrading CPF from aqueous phase. Maximum biodegradation of CPF occurs beyond pH 7 where in which, hydrolysis also plays a dominant role. The biodegradation of CPF is strongly influence by the density of Actinomycetes sp. and its prevailing concentrations in aqueous phase. Complete and total biodegradation of CPF takes place beyond 2 d and at pH 7. Actinomycetes sp. can prefer precipitated form of CPF than the dissolved form. The degradation of CPF appeared to follow simple first-order kinetics. The specific growth rate vary between 0.09 and 0.54 1/h with a maximum value of 0.08 1/h and an average endogenous decay coefficient of 0.016 1/h under the CPF concentrations varying between 1 and 8 mg/l. Lindane toxicity pressure occurs both in monoculture and in mixed culture media.

REFERENCES