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# Biodegradation of Pesticide Chlorpyrifos by Bacteria *Staphylococcus aureus* (Accession no. CP023500.1) Isolated from Agricultural Soil

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## Abstract

The use of pesticides like Chlorpyrifos in agricultural soil is the primary reason for the pollution of aquatic and terrestrial environments. Today the most effective method used for bioremediation are by using microbes. Different pesticide degrading bacteria were isolated and identified by the mean of cultural, biochemical tests and which is further identified and confirmed by 16S RNA sequencing method. The most potent strain S-1 growth in mineral salt medium supplemented with Chlorpyrifos as sole source of carbon (50 to 1000 ug/ml) its optical density was measured at 600 nm. The bacterial growth is optimised on the parameter of different physio-chemical condition were. The result showed that S. aureus shows maximum growth on 12$^{th}$ day. The HPLC analysis was also done for calculating the residual percentage of Chlorpyrifos after 12 days incubation which showed that *S. aureus* was able to degrade 99% of the pesticide of the 1000 ug/ml CP concentration in the MSM. The results of this research shows that the isolated bacteria have the potential to be used in bioremediation of Chlorpyrifos contaminated soil and water ecosystems.

**Keywords:** Bacterial, Bioremediation, Chlorpyrifos, Degradation, Pesticide

## 1. Introduction

The 70% of total population of India is dependent on the agricultural primarily which is the maximum portion of the country's economy[1]. Pesticides are varied and large group of substances used for killing the harmful organisms like weeds, insects, rodents among others. The extensive use of such pesticides results in the accumulation of pesticide in our atmosphere. Many of these pesticides can persist in the soil and they can also contaminate the surface and the ground-water. The Organophosphate (OP) insecticides like Chlorpyrifos insecticide is also widely used and can lead to contamination of soil and water bodies. This pesticide is having a broad-spectrum range and it is extensively used in the prevention of agricultural pests and it is moderately toxic insecticide. In the environment the Chlorpyrifos has been reported as having from 10 to 120 days of half-life in various soil and major degraded non-toxic end product is 3,5,6-trichloro-2-pyridinol (TCP). The high-level of the end product like TCP don't support the growth and the proliferation of soil-microorganism capable of degrading the Chlorpyrifos. The microorganism having the potential to degrade the pesticide are capable of releasing the metabolic enzyme in the soil helping in bioremediation of many anthropogenic chemicals[2]. Bacteria with the capability to utilise the pesticide organophosphate have been isolated and characterized from the soil all over the world[3], Pseudomonas aeruginosa isolated from the soil is the gram negative bacteria and is the most common bacteria with the potential to degrade Chlorpyrifos[4]. In the present study the pesticide Chlorpyrifos is taken for bioremediation under different physiochemical conditions. The main objectives of this present study are to isolate the contaminated soil bacterial strains and further to characterize its Chlorpyrifos degradation potential in the MSM consisting of Chlorpyrifos as an only source of carbon in different concentrations and other environmental and chemical factors.

## 2. Materials and Methodology

### 2.1 Chemicals

All highest purity grade chemicals were used and obtained from the HiMedia, Merck and Qualigens.

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### 2.2 Sample Collection

Agricultural soil were taken from different area of Patna, Bihar which have for the past few years had exposure history of Chlorpyrifos were selected for this study. Samples were collected randomly from three rice fields and three other crops fields from 12–15 cm depth of the field and stored aseptically for further analysis.

### 2.3 Isolation of Chlorpyrifos Degrading Bacteria

For isolation of microbes from different soil samples serial dilution were carried out by dissolving 0.1 g of each soil samples in 9.9 ml of normal saline solution.

For isolation of bacteria 1 ml of soil suspension of different samples were spread over the pre sterilized petriplates containing nutrient agar media at dilution $10^{-5}$, $10^{-6}$ and $10^{-7}$ and further the plates were incubated at ambient temperature of 37ºC for 24-48 hrs.

The enrichment nutrient culture media technique was used for isolation of the Chlorpyrifos utilizing microbes present in the soil samples, in which the pesticide Chlorpyrifos is used as a only carbon source in different concentration. This was done by sub-culturing those pure cultures of bacteria and fungi on pesticides containing NA media plates and incubated at 37ºC for bacterial plates[5].

### 2.4 Characterisation and Identification of Isolates

The isolated bacterial obtained on the Chlorpyrifos agar solid plate were further characterised by different biochemical tests like; gram staining, citrate utilization, catalase test, citrate utilization, sugar fermentation, oxidase test, motility, Voges-Proskeur test, methyl-red test, nitrate reduction, starch hydrolysis and hydrogen sulphide production. The purified bacterial culture were identified according to the Gerhard et al. and then using Bergey's Manual of Determinative Bacteriology for confirmation[6].

The best selected strain with maximum degradation ability was further confirmed as per the result of 16s rRNA sequencing at Yaazh Xenomics (Madurai, Tamil Nadu, India). The phylogenic neighbor-joining tree was using complete 16S rRNA gene sequence analysis.

### 2.5 Biochemical Tests

Some biochemical tests were conducted to show the ability of the isolates to produce enzymes such as catalase, oxidase, coagulase etc. Tests were conducted to check their ability to utilize citrate, reduce nitrate, produce indole etc. in addition to methylene red tests[7].

### 2.6 Effects of Various Physico-chemical Parameters

Several physical and chemical parameters were used for optimizing the isolated bacterial growth which includes: Incubation temperature, pH, salt concentration, carbon-source availability, nitrogen source, affecting bacterial-growth were considered[8].

### 2.7 Growth Kinetic of Bacterial Isolates at Different Concentration of Compounds

Growth curve experiments were performed with different doses of Chlorpyrifos compounds in order to determine the optimum concentration of that stimulates the growth of isolates in liquid medium at different concentration (i.e. 200 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml) of at interval of 2, 4, 8, 12 and 14 days using spectrophotometer (Rani et al. 2008). After incubating at optimum condition, the Chlroprifos (CP) the degradation and residual percentage of CP was calculated by using HPLC analysis[9].

## 3. Results

### 3.1 Isolation and Characterization of Chlroprifos (CP) Degrading Bacteria

From the agricultural soil, the bacterial cultures were isolated on by the help of MSM containing CP in different concentration (50 to 1000 ug/ml). 35 different bacterial cultures were obtained from different soil sample. After initial screen 15 with good potential of degradation of CP were selected for further study, in which the strain S-1 was selected as the best degrader strain and shows higher degradation at 1000 ug/ml of CP concentration.

### 3.2 Cultural and Biochemical Characteristics of the Selected Isolate

The bacteria strain, S-1 on solid NA (Nutrient Agar) plate shows yellow colour, smooth and circular with regular margins as shown in (Figure 1). Its microscope view and the gram's staining shows it as coccus in cluster and gram-positive, coccus in cluster (Figure 2). The biochemical tests performed on the isolate showed the positive results for catalase, citrate, coagulase, sucrose and glucose fermentation with no gas formation and MR tests. As per the Bergey's Manual of Systematic Bacteriology (Gerhard et al., (1981)), the strain S-1 was identified up to the genus level as *Staphyloccus* species and it was further confirmed by the help of 16S rRNA sequencing, as *Staphylococcus aureus* (Accession no. CP023500.1) at Yaazh Xenomics, Madurai, Tamil Nadu (India) with an Accession No. CP023500.1. The phylogenetic Neighbor-Joining tree

(Figure 3) was constructed for strain S-1 using an almost complete 16S rRNA gene sequence (784 bp).

By using NCBI blast similarity search tool the 16s rRNA sequence was constructed. The MUSCLE 3.7 program was further used for the multiple alignments of sequences[10] which was further cured by using Gblocks 0.91 b. in to a aligned sequence[11]. The program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model and the programmed Tree Dyn 198.3 was used for tree construction[12].

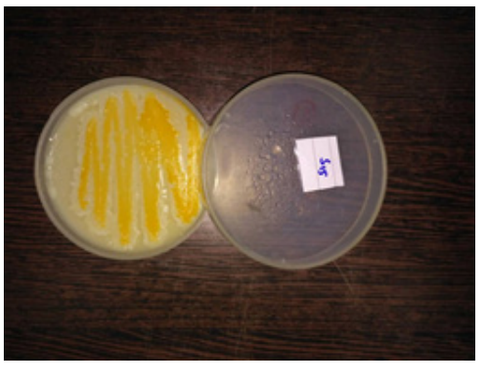

**Figure 1.** Coloney characteristics on NA plate

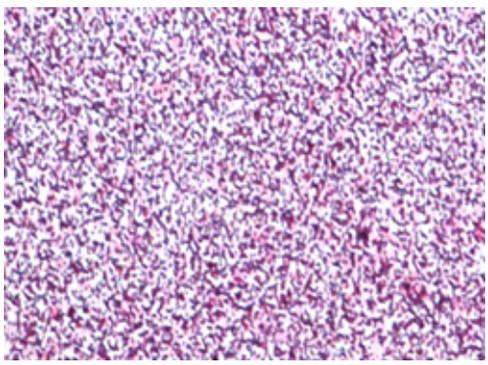

**Figure 2.** Microscopic view (under 40 X).

## 3.3 Effect of Different Physiochemical Parameters on Growth of Isolate S1 (Ph, Temperature, NaCl, Carbon and Nitrogen Concentration)

To optimise the further physical and chemical characteristics the selected bacteria culture was subjected to different environmental condition like pH (3–10), temperature (6–50°C) and NaCl concentrations (1–10 %), Carbon source like Glucose, Lactose, and Sucrose (1 to 5 %) and Nitrogen source like Peptone, Yeast extract, Beef extract and Casein (1 to 5 %). The observations show that S-1 (S. aureus) can tolerate a wide range of pH from low acidic to highly alkaline condition which shows its pH adaptability with the best growth at pH 6-7. It was also found that the S1 was able to survive well even at high temperature of 50°C and also shows tolerance to wide temperature range from 6–50°C, with the best growth at 37°C, it was observed that the growth was further restricted at higher temperatures from 50°C onwards. The tolerance range of halophytic environment condition is also found of wide range and the S-1 was capable of growing from the range of 1-20 % NaCl concentrations and the isolate was found to highly halophytic in nature and was able to grow even at high concentration of 20% of NaCl. The data are presented in (Table 1). Among the different carbon source the shows its best growth in the presence glucose at 5% (Table 2) and Yeast extract as best Nitrogen source (1%) as shown in (Table 3). As reported the soil in the India varies largely in its proprieties like from acidic to alkaline through neutral, salinity and other properties. The study and degradation potential of the

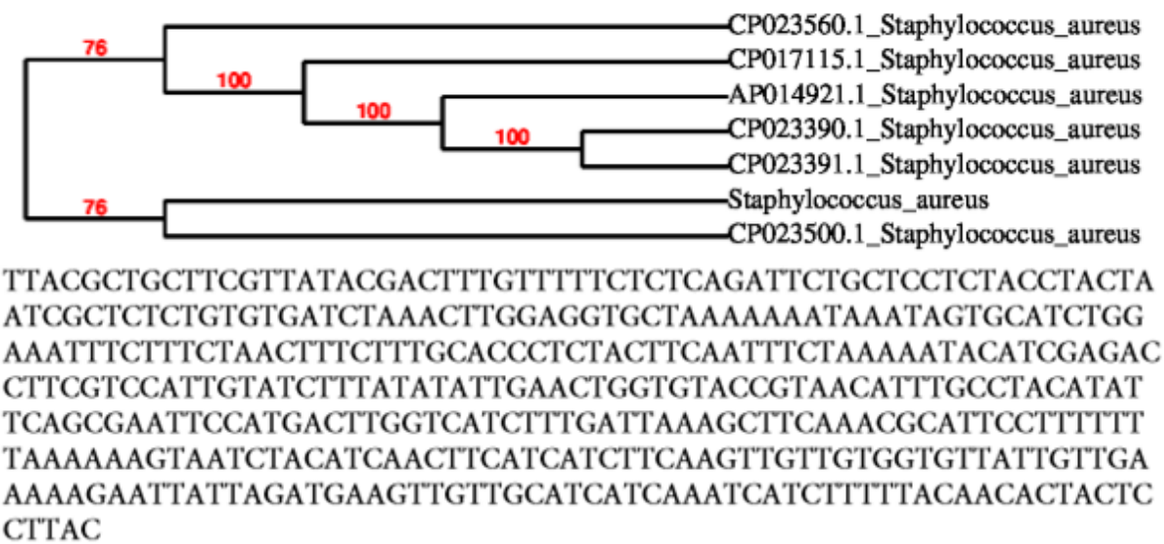


**Figure 3.** Phylogenic analysis of strain S-1 based on 16s r RNA analysis. The sequence bar equals 0.02changes per nucleotide position.

**Table 1.** Effect of different environmental condition in growth of isolates S-1

| pH | Growth | Temperature | Growth | NaCl% | Growth |
|---|---|---|---|---|---|
| 3 | - | 6 | ++ | 1 | ++ |
| 4 | ++ | 12 | ++ | 5 | +++ |
| 6.5 | +++ | 27 | ++ | 10 | ++ |
| 10 | ++ | 37 | +++ | 15 | ++ |
| 12 | - | 50 | ++ | 20 | ++ |

**Table 2.** Effect of different carbon source on growth of isolates S-1

| Concentration | Glucose | Lactose | Sucrose |
|---|---|---|---|
| 1% | ++ | +++ | - |
| 2% | +++ | +++ | ++ |
| 3% | ++ | +++ | ++ |
| 4% | ++ | ++ | ++ |
| 5% | +++ | ++ | ++ |

strain having high salinity and pH tolerance will help in the bioremediation study of Indian soil.

### 3.4 Growth Response of *Staphylococcus aureus* and HPLC Analysis

The selected strain S1 was subjected to different concentration of CP (50 to 1000 ug/ml) as sole source of Carbon in MSM media for different time interval. The growth kinetics shows that the isolated was able to degrade the CP even at higher concentration as 1000 ug/ml and (Figure 4) shows that the isolate uses the CP as the sole energy and carbon-source. The best growth of S. aureus was reported on the 12 days of incubation.

**Table 3.** Effect of different nitrogen source on growth of isolates S-1

| Concentration | Yeast Extract | Beef extract | Peptone | Casein |
|---|---|---|---|---|
| 1% | +++ | - | ++ | - |
| 2% | +++ | ++ | +++ | - |
| 3% | +++ | ++ | +++ | ++ |
| 4% | ++ | ++ | ++ | - |
| 5% | ++ | ++ | ++ | - |

"- = No growth, ++ = moderate growth, +++ = luxuriant growth, initial medium pH 7."

By the HPLC-analysis, the degradation percentage was reported of the strain S-1, the chromatograms of the degraded pesticides are shown in (Figure 5). The reduction percentage and the Chlorpyrifos concentration are reported in (Table 4). High degradation percentage of 99% was achieved.

## 4. Discussion

The results obtained in this study were found in the agreement with the previous reports which shows that the degradation of Chlorpyrifos was observed at 30 where as in our result culture is capable of growing at wide range of temperature however most luxuriant was 30 to 37 °C.[13]. It was reported that the optimum physical and chemical condition plays an import role in accelerating the degradation of Chlorpyrifos[14]. It was reported that the bacteria like *Sphingomonas* sp. have the potential to use Chlorpyrifos as a carbon and energy sole source for its growth, by breaking down the Chlorpyrifos into the simple compund 3,5,6-trichloro-2-pyridinol[15].

Many authors reported that the most species specially the Gram negative bacteria, like *Enterobacteriaceae* sp. have the property to degradation of insecticides like Chlorpyrifos, but

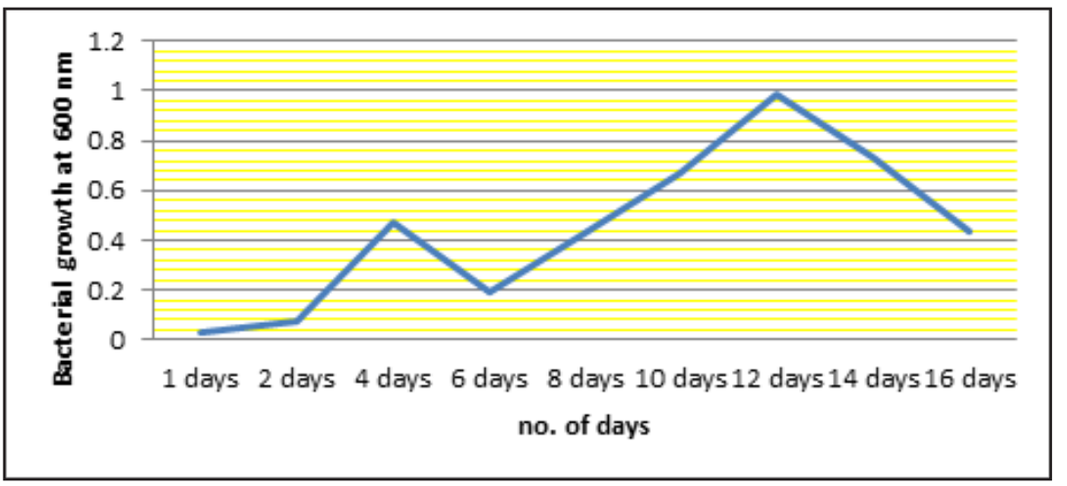


**Figure 4.** Growth kinetics of isolates S-1 at concentration of 1000 ug/ml.

**Table 4.** Degradation of CP by isolate S1

| Code | Spike Sample (µg/ml) | Dilution Factor (DF) | HPLC area of dillution | Concentration of dilution found (µg/ml) | Concentration of Sample (C*DF) | % Degraded |
|---|---|---|---|---|---|---|
| S1 | 1000 | 1 | 396820 | 0.960430466 | 0.960430466 | 99.90 (approx.) |

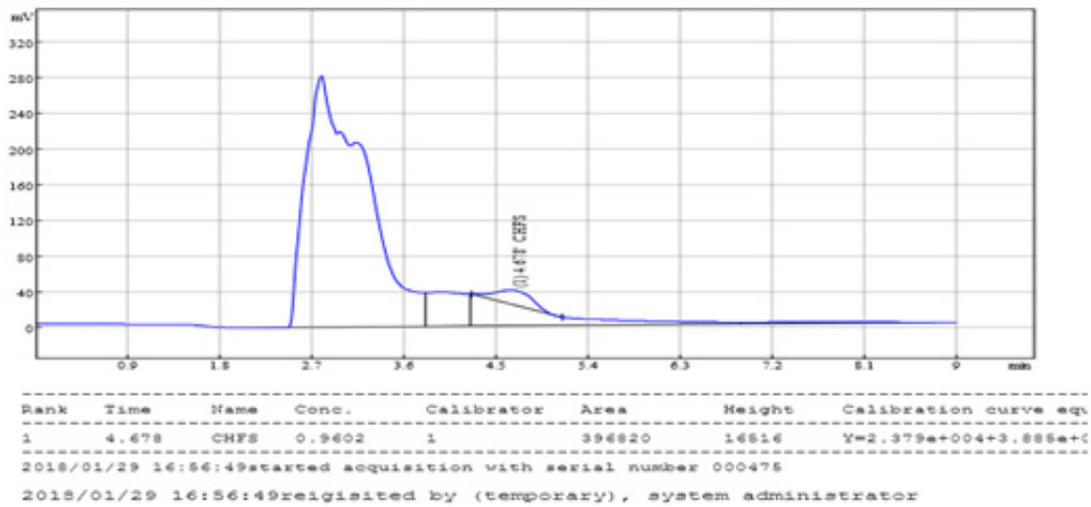


**Figure 5.** HPLC chromatogram of CP after degradation by S-1.

many other reports also shows that the many gram negative bacteria have the property of degradation the pesticide but they show the degradation upto only 60%. Many degrader bacteria present in the soil were reported with the capability to degrade the pesticides, among all the bacteria *Pseudomonas aeruginosa* was the most common reported bacteria strain, it is a gram negative bacterium present in the soil and is also have the potential to utilise the Chlorpyrifos, other CP degrading reported bacteria are *Providencia* sp *Serratia* sp, *Bacillus* sp and *Klebsiella* sp, capable of degradation of Chlorpyrifos[16,17].

The Chlorpyrifos degradation percentage was also reported in other studies like the rate of degradation of Chlorpyrifos by bacteria *Alcaligenes faecalis* DSP3 was approx 76.2% in the MSM maintained at pH 7 and temperature 30°C for around 18 days. The further study also reports the isolates like *Bacillus pumilus* C2A1 with degradation with the degradation potential of around 89% of 1000 mg l-1 in around 15 day[18]. As per result obtained in our study the degradation percentage is as high as 99% and the isolate S-1 also show ability to grow in vast range of physiochemical condition.

## 5. Conclusion

The results obtained in our study shows that bacterial isolates is having a very important role in the bio-degradation of pesticides with the potential of bio-remediation. In this study S1 is having the potential of degrading the pesticide CP and using it as a energy and carbon source. The selected strain S-1 was further identified and characterization as a highly halophylic in nature and can tolerate wide range of pH also. The bioremediation and degradation of the toxic pesticides by mean of bacteria is one of the most successful and effective method of preventing environmental pollution. The result of our study shows that the isolates *Staphylococcus aureus* is having high degradation potential and can convert upto 99% of Chlorpyrifos from the medium and have a high potential of bioremediation of Chlorpyrifos from the contaminated agricultural soil or other contaminated environment.

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