



## Biodegradation Of Residual Chlorpyrifos By Naturally Occurring Bacteria

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### ABSTRACT

The indiscriminate use of Chlorpyrifos is one of the major causes of its ubiquitous presence as a contaminant in soil and water environment. Biodegradation process is a promising method to remove such contaminant from polluted area. In the present work, four Chlorpyrifos degrading bacteria namely *Micrococcus varians*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Micrococcus luteus* were isolated, screened on Mineral Salt Medium containing 25mg/L of Chlorpyrifos concentrations as well as identified through cultural and biochemical tests. The optimum conditions were also examined which found to be varied between 30°C to 37°C and pH 7 to 8. All of the tested bacteria showed capability of utilizing 25-100mg/L of Chlorpyrifos at 96hrs of incubation period. Among the four tested isolates, the growth of *Pseudomonas aeruginosa* was found maximum at all tested culture conditions. Therefore, the results of this research indicated that the isolated bacteria can be exploited for bioremediation of Chlorpyrifos contaminated compartments.

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## INTRODUCTION

Enhanced industrial and agricultural activities throughout the world depend on a variety of synthetically produced chemicals that has led to considerable pollution of environment. Pollution of soil and water by agro-chemicals is an emerging problem in agriculture based countries since various types of hazardous and non-hazardous chemicals are continually being applied in agricultural lands, the major proportion of which is not utilized, and eventually dissipated into the environment. Pesticide is defined as any substance or mixture of substances intended for repelling, preventing, destroying, or mitigating any pest such as insects, weeds, mites, nematodes, rats, etc. (Arias-Estévez et al., 2008; Javaid et al., 2016). Pesticides include insecticide, herbicide, fungicide, and various other substances (Zhang et al., 2011). Various types of pesticides, such as organochlorine, organophosphate, carbamate, pyrethroid etc. are routinely applied during agricultural activities (Chowdhury et al., 2012). Organophosphorus forms a major group of pesticides which is account for about 36% of the total pesticides used globally (Briceño et al., 2012; Xie et al., 2010). It is ironic that only less than 0.3% of the applied pesticides are reached to the target organisms, and the rest that is thereafter referred to as residual, remain in the top of the soil, and the ultimate result leads to pollution of soil and water (Hossain et al., 2015; Van der Werf, 1996).

An organophosphate pesticide Chlorpyrifos (CP), which has the chemical name O, O-diethyl O-3, 5, 6-trichloropyridyl phosphorothioate and the trade names Lorsban, Agromil, Dhanwan, Dorson, Omexan, Dursban, Suscon Green, Empire, Equity has been widely used globally since 1965 as an insecticide to control crop pests in agriculture, mosquito as well as soil dwelling grubs, rootworms, borers and subterranean termites (Anwar et al., 2009; Chen et al., 2012; Lee et al., 2012; George et al., 2014; Singh et al., 2004). CP adsorbs strongly to soil particles and it is not readily soluble in water (Díaz-Cruz and

Barceló, 2006). It is therefore immobile in soils and unlikely to leach or to contaminate groundwater. The principle metabolite of CP, 3, 5, 6-trichloro-2-pyridinol (TCP), adsorbs weakly to soil particles and appears to be moderately mobile and persistent in soils (Li et al., 2007). The half-life of CP in soil usually varies from 65 to 360 days that can also be over 1 year, depending on the soil type, climate, and other conditions (Chen et al., 2012). CP is acutely toxic to bees, birds, mammals, aquatic life, and certain species of algae. Poisoning from CP in human may affect the central nervous system, the cardiovascular system, and the respiratory system as well as it is a skin and eye irritant (Chen et al., 2012; Pailan et al., 2016). Acute exposure can result in such symptoms as numbness, tingling sensation, incoordination, dizziness, vomiting, sweating, nausea, stomach cramps, headache, vision disturbances, muscle twitching, drowsiness, anxiety, slurred speech, depression, confusion and in extreme cases, respiratory arrest, unconsciousness, convulsions, and death (Lee et al., 2012). It is therefore crucial to neutralize or detoxify the residual CP in the agricultural lands, which otherwise remain in the environment for an extended period of time. Several methods have been adapted for the treatment of polluted area. These include chemical treatment, volatilization and incineration, which have met public opposition because of the use of large volume of chemicals and for the high cost (Eissa et al., 2014; Jabeen et al., 2015). Due to such constraints, alternative treating techniques, such as those using microbial processes are gaining more considerations, while naturally occurring microorganisms break down pesticides during their metabolic activities by a process known as bioremediation that is cost-effective and eco-friendly (Ajaz et al., 2012; Abraham and Silambarasan, 2013).

The aim of present research was to isolate, characterize and identify CP degrading native strains of bacteria, assessment of growth

potential in presence of CP and finally to investigate the effect of temperature and pH at varying interval of time and CP concentration on the growth response of the selected bacterial isolates.

#### **MATERIALS AND METHODS:**

##### ***a. Sample collection***

Soil samples were collected about 15 cm below the soil surface using a sterile spatula from multiple points of some paddy fields located in the Hathazari area of Chittagong. Samples were collected in sterile plastic bottles and preserved at 4°C for further analysis.

##### ***b. Isolation and selection of CP degrading bacterial strains***

Soil suspension was prepared by mixing 10gm. of sieved soil in 90ml distilled water. Serial dilutions of the suspension upto  $10^{-6}$  were performed in order to achieve single colony of pure culture. For the isolation of bacteria, pour plate method using nutrient agar medium was used. Morphologically distinguishable bacterial colonies were picked up and streak further on nutrient agar plate for purification. In case of no single colony appeared, repeated streaking from plate to plate was carried out until multiple single colonies were found. The isolated strains were stored at 4°C on nutrient agar slant. Occasional sub-culturing at 3-week interval was regularly maintained to keep characters unimpaired.

##### ***c. Screening of microbes***

The inoculum for all of the experiments were prepared by growing bacteria in separate LB broth, pH 7 at 37°C and incubated for time required to obtain mid logarithmic cells of density  $10^6$ /ml on a rotary shaker at 120rpm and measuring OD at 625 nm in a UV-visible spectrophotometer. 2ml from each inoculum prepared following above mentioned method were inoculated into separate Erlenmeyer flask containing Minimal Salt Medium (MSM) supplemented with 25mg/L CP, incubated for 5days at shaking condition of 37°C and the growth of bacteria were calculated from the values of OD at 560nm of the culture supernatant following centrifugation at 4000

rpm for 10 minutes. Control was maintained at each step.

##### ***d. Characterization of bacterial isolates***

Pure bacterial strains were identified on the basis of microscopic tests, morphological and physiological characteristics in nutrient agar plates and slants. Colony morphology, Margin, Form, Texture, Elevation and Color were studied. Later for the confirmative test, biochemical tests including motility test, indole production test, methyl red-Voges Proskeur (MR-VP) test, Simmons' citrate test, catalase test, oxidase test, glucose fermentation test, lactose fermentation test, nitrate reduction test, H<sub>2</sub>S production test, and urease test were carried out.

##### ***e. Optimization of culture conditions:***

**pH:** In order to identify the optimal pH for selected bacterial growth, MSM containing 25mg/L of CP was taken in separate Erlenmeyer flask. Following sterilization and cooling the medium was inoculated with inoculum containing  $10^6$ /ml cells of each organism in respective flask and maintained at different pH (4, 5, 6, 7 and 8). After incubation at 37°C temperature for 5days, 2ml of culture was drawn and centrifuged at 4000rpm for 10 minutes. The pellet was discarded and the supernatant was collected to evaluate the growth of CP degrading bacteria and the optical density was taken at 560nm using UV-visible spectrophotometer. Control was maintained at each step. (Kavikarunya, 2012)

**Temperature:** To identify the optimal temperature required for the best growth of isolated bacteria, MSM containing 25mg/L of CP was taken in Erlenmeyer flask. Following sterilization and cooling the medium was inoculated with inoculum containing  $10^6$ /ml cells of each organism and maintained at different temperature (20°C, 30°C, 37°C, and 45°C). After incubation for 5days, 2ml of culture was drawn and centrifuged at 4000rpm for 10 minutes. The pellet was discarded and the supernatant was collected to evaluate the growth of CP degrading bacteria and the optical density was taken at 560nm using UV-visible

spectrophotometer. Control was maintained at each step. (Kavikarunya, 2012)

**Chlorpyrifos concentration:** To identify the concentration of CP upto which the selected bacteria show resistance, different concentration (25mg/L, 50mg/L, 75mg/L and 100mg/L) of CP were added to separate Erlenmeyer flask containing MSM. Following sterilization and cooling the medium was inoculated with respective inoculum containing  $10^6$ /ml cells of each organism and maintained at 37°C and pH7. After incubation for 5days, 2ml of culture was drawn and centrifuged at 4000rpm for 10minutes. The pellet was discarded and the supernatant was collected to evaluate the growth of CP degrading bacteria and the optical density was taken at 560nm using UV-visible spectrophotometer. A control was maintained at each step.

**Incubation period:** Minimal salt broth supplemented with 25mg/L CP was taken in separate Erlenmeyer flasks. Following sterilization and cooling the medium was inoculated with inoculum containing  $10^6$ /ml cells of respective test organisms. The cultures were incubated for 120 hours at 37°C and pH7. For the analysis of bacterial growth, 2ml culture from each flask were withdrawn at regular intervals of 24hrs and each for five continuous days and growth was measured at OD560.

#### RESULTS AND DISCUSSION:

From time to time, diverse physical, chemical and biological treatment systems, either independently, subsequently one after another, or in concert, have long been practiced as effective measures in dealing the hazardous environmental contaminants. As physicochemical methods are often expensive and laborious, the efficient, cost-effective, simple, and more importantly, environmentally friendly biodegradation processes are becoming attracting scientists' interest as novel

procedure to treat natural matrices polluted with various contaminants.

Twenty naturally occurring bacterial colonies coded as AS1, AS2, BS1, BS2, BS3, BS4, BS5, BS6, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS8, CW1, CW2, CW3, and CW4 were isolated from the pesticide contaminated soil of paddy fields, which were more or less capable of detoxifying the concentration of CP. The bacterial isolates were obtained from Hathazari area of Chittagong, which is having a repeated history of pesticide application. The microbial communities in this area were exposed to pesticide application for past many years, which resulted in adaptation of these microbes against CP.

Four strains coded as AS1, AS2, BS2, and BS3 whose actions were reflected in significant growth were selected for further study via screening procedure using MSM containing CP which enabled us to compare the growth efficiency of the isolates and find out the best isolates. The result of screening of fifteen potential isolates is shown in Fig. 1. In the present study, inoculum size of  $10^6$  cells/ml was maintained in each step which was recommendable to use to detoxify pesticide polluted area (Anwar et al., 2009). CP was being artificially added in the MSM with no carbon source which proved that the microbes utilize CP as alternative carbon source (Ajaz et al., 2012). Utilization of CP as sole source of carbon indicated that the pyridinyl ring of CP had been cleaved and degradation of CP was occurred (Rayu et al., 2017).

For the identification of screened bacteria, the observed microscopic, cultural and biochemical characteristics of the strains were compared with the standard descriptions of "Bergey's Manual of Determinative Bacteriology". The results obtained are shown in Table-1 and Table-2.

*Table-1: Physical and morphological characterization of the bacterial isolates*

Bacterial Isolates	Form	Margin	Elevation	Surface	Opacity	Pigmentation
AS1	circular	Entire	convex	Smooth	Opaque	Yellowish white
AS2	circular	Entire	Flat	Smooth	Opaque	Red
BS2	circular	Entire	convex	Smooth	Translucent	Yellow
BS3	circular	Entire	convex	Smooth	Opaque	Yellow

*Table-2: Biochemical characterization and identification of CP-resistant bacterial isolates*

Name of the Tests	Behavior of Isolates			
	AS1	AS2	BS2	BS3
Gram Reaction	+	-	-	+
Morphology	Cocci	Short Rod	Rod	Cocci
Motility	-	+	+	-
Indole	-	-	-	-
Voges-Proskauer	-	+	-	-
Methyl-red	-	-	-	-
Simmons' Citrate	-	+	+	-
Nitrate Reduction	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	-	+	+
Urease	+	-	-	+
Glucose Fermentation	+	+	-	-
Lactose Fermentation	-	-	-	-
H <sub>2</sub> S Production	-	-	-	-
Identification	<i>Micrococcus varians</i>	<i>Serratia marcescens</i>	<i>Pseudomonas aeruginosa</i>	<i>Micrococcus luteus</i>

Note: + : Positive, - : Negative

Both Gram positive and Gram negative bacteria were obtained in this study which were involved in CP degradation. Previous studies have reported the degradation of CP using various bacteria such as *Enterobacter* sp. (Singh et al., 2004), *Alcaligenes faecalis* (Yang et al., 2005), *Sphingomonas* sp. (Li et al., 2007), *Paracoccus* sp. (Xu et al., 2008), *Bacillus*

*pumilus* (Anwar et al., 2009), *Gordonia* sp. (Abraham et al., 2013), *Sphingobacterium* sp. (Abraham and Silambarasan, 2013), *Cellulomonas fimi* (Barathidasan et al., 2014), *Acinetobacter* sp. (Pailan et al., 2016) etc. However, to our knowledge, for the first time this report showed the degradation of CP by

*Micrococcus varians* AS1 and *Micrococcus luteus* BS3.

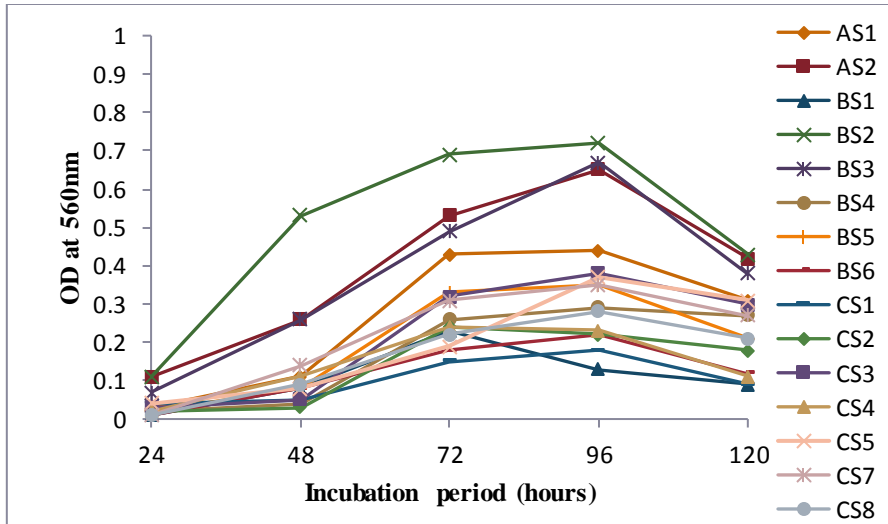
One of the most important factor that influence pesticide biodegradation processes is soil pH (Nicholls, 1988). The selected bacteria were able to grow and degrade CP in all pH values studied (pH 4.0-8.0). The growth of bacteria was faster at high pH value i.e at pH 7.0 and 8.0 than low pH i.e at pH 4.0 in all cases. This study is similar to the experiment of Anwar et al. (2009), who reported maximum degradation of CP at higher pH value. It is may be due to the reason that some enzymes responsible for CP degradation have their optimum activity at high pH (Anwar et al., 2009). Racke et al. (1996) and Singh et al. (2003) also reported the hydrolysis of CP at high pH. The maximum growth rate of three bacteria namely *Serratia marcescens*, *Pseudomonas aeruginosa* and *Micrococcus luteus* was observed at pH 8.0 while the highest growth rate of *Micrococcus varians* was recorded at pH 7.0. It is also observed that, better growth of *Pseudomonas aeruginosa* was found among the four bacterial isolates followed by *Micrococcus luteus*, *Serratia marcescens* and *Micrococcus varians*, respectively. The result obtained is depicted in Fig. 2.

Temperature is an important environmental factor that influences the growth of bacteria under different culture conditions. In the present work, the growth of selected CP degrading bacteria was assessed in various temperatures viz., 20°C, 30°C, 35°C and 45°C. The growth rate of all tested bacteria was found

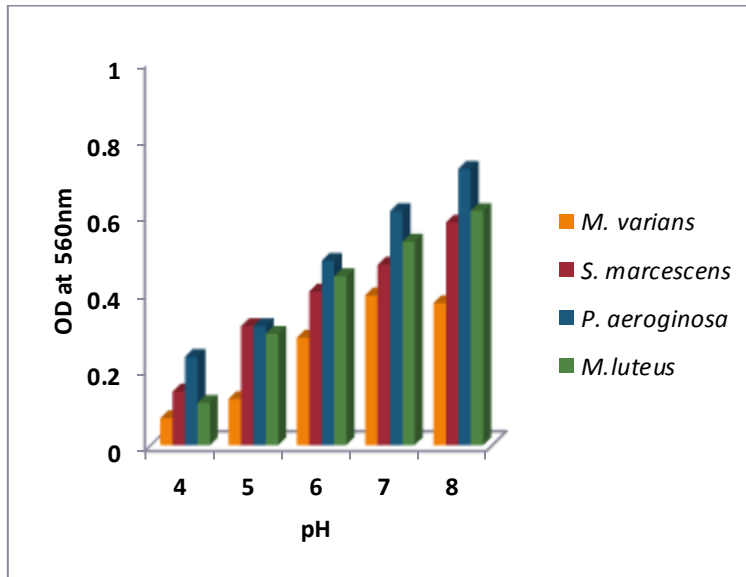
maximum at 37°C. There was a little fluctuation in the growth of bacteria when the temperature decreased from 37°C to 30°C but sharp decrease observed when the temperature was below 30°C or increased from 37 to 45°C. Among four bacterial isolates, *Pseudomonas aeruginosa* was promising for CP removal as it shows better growth at all tested temperature. (Fig. 3)

The effect of incubation period on the growth of selected CP degrading bacteria shows in the Fig. 4. The growth rate of all tested bacteria was increased consistently with time and a dramatic increase was observed at 72hrs whereas after 96hrs the growth of bacteria was drop down and reduction in the growth was observed. The growth was maximum at 96hrs followed 72hrs. *Pseudomonas aeruginosa* showed better growth at different incubation periods as compared to other three isolates.

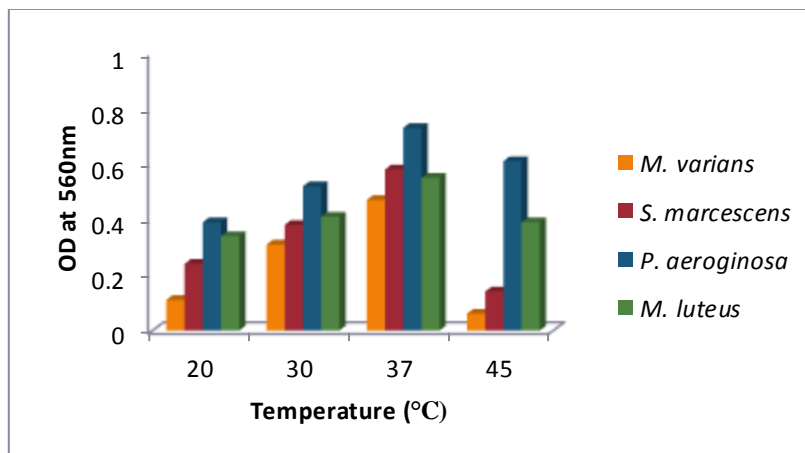
The obtained data clearly revealed that, with the increasing of CP concentration from 25 to 75 mg/L, the growth rate of tested bacteria was gradually decreased (Fig. 5). *Pseudomonas aeruginosa* and *Micrococcus luteus* showed better growth at all concentration level as well as they showed tolerance at concentration 100mg/L comparing to other bacteria. Investigations with different CP concentrations in other experiments also reported higher degradation efficiencies at lower CP concentrations (Rani et al., 2008; Vijayalakshmi and Usha, 2012; Yadav et al., 2014).



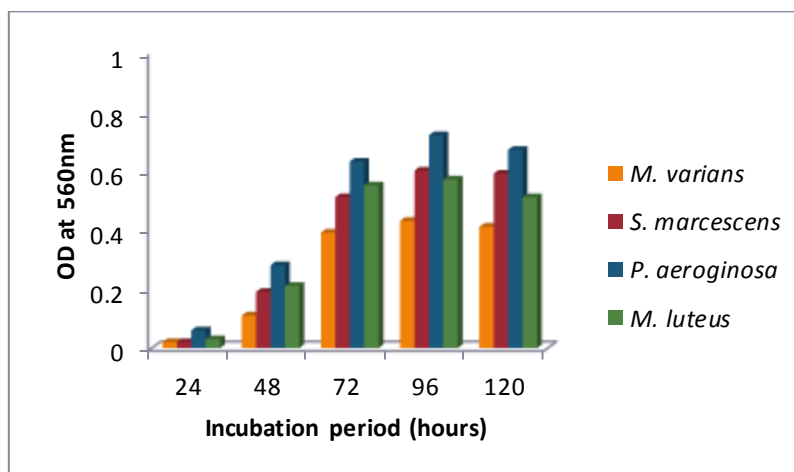
*Fig. 1. Bacterial isolates from paddy fields exhibiting significant growth. The experiment was conducted by inoculation of MSM supplemented with 25 mg/L of CP, incubation at 37°C for 7 days and determination of growth as measured the absorbance at 560 nm in a UV-visible spectrophotometer.*



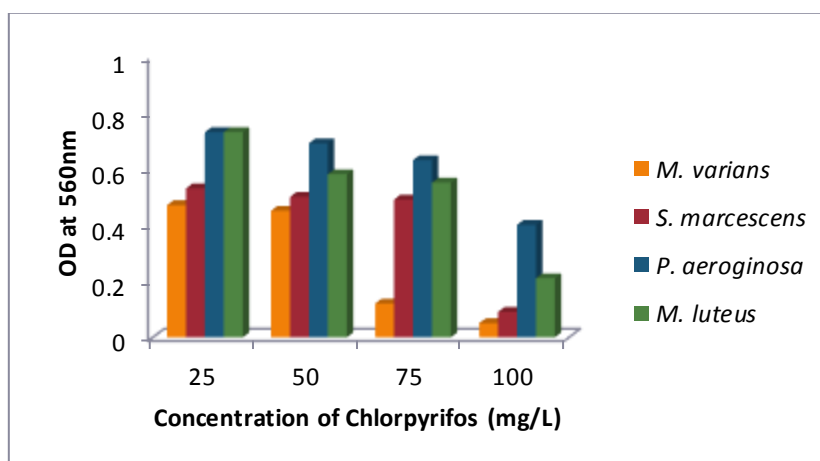
*Fig. 2. Effect of pH for the growth of CP degrading bacteria in Minimal Salt broth supplemented with 25mg/L of CP*



*Fig. 3. Effect of temperature for the growth of CP degrading bacteria in Minimal Salt broth supplemented with 25mg/L of CP*



*Fig. 4. Effect of incubation period for the growth of CP degrading bacteria in Minimal Salt broth supplemented with 25mg/L of CP*



*Fig. 5. Effect of CP concentration for the growth of CP degrading bacteria in Minimal Salt broth supplemented with 25mg/L of CP*

#### CONCLUSION:

Isolation and characterization of CP degrading microorganisms are important in order to identify variety of mechanisms involved in enhance degradation of CP. This research concludes that *Micrococcus varians*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Micrococcus luteus* may possess potential to be used to detoxify CP polluted environment. However, extensive research is required to identify the genes and enzymes and to elucidate the exact degradative pathways involved in microbial degradation of CP to save our green planet for our next generation.

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