# DEGRADATION OF HERBICIDE (3-CHLOROPROPIONIC ACID) BY BACTERIAL DEHALOGENASES

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## ABSTRACT

3-Chloropropionic acid (3CP) is a synthetic halogenated aliphatic compound and is part of the active ingredients used in certain herbicides. Since not many β-chloro substituted haloalkanoates were given attention as their counterparts (α-chloro substituted haloalkanoates), and not many bacteria were reported as capable of utilizing this toxic compound as their sole carbon source. In current investigation, a bacteria isolated from padi field was identified as Rhodococcus sp. using 16S rRNA technique. The bacterial species was able to utilize 3CP as sole source of carbon and energy. This finding was supported by the growth analysis in 20 mM 3CP minimal medium with cell doubling time of 12.50 hours.

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## INTRODUCTION

Various xenobiotics have been produced due to the advancement in industrial technology and intensification of agricultural practices. These compounds have been released into the environment in large quantities and their resistance to biodegradation had lead to the accumulation of the halogenated compounds in the environment. The use of herbicides in general (2,2-dichloropropionic acid- Dow Chemical-Dalapon or 3-chloropropioniac acid) are the main contributor of environmental pollutions.

Growth of bacteria in flouroacetate, monochloroacetate, trichloroacetate, 2-chloropropionate and 2,2-dichloropropionate (2,2DCP) have been documented (Allison *et al.*, 1985; Goldman, 1965; Hardman and Slater, 198; Kurihara *et al.*, 2000; Kurihara *et al.*, 2003; Yu and Welander, 1995). Microorganisms utilizing ß-substituted halogenated compound such as 3-chloropropionate, 3-bromopropionate and 3-iodopropionate were not well studied. Some of the microorganisms which have been shown to dehalogenate 3CP are *Micrococcus denitrificans* (Bollag and Alexander, 1971) and *Clostridium kluyveri* (Hashimoto and Simon, 1975). However, dehalogenases from these microorganisms are not well characterized. Therefore, this study was carried out to study a new bacterial isolate that can degrade 3-chloropropionic acid (Figure 1).

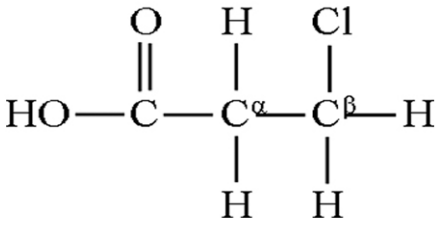


Figure 1: Structure of 3-chloropropionic acid (3CP)

## MATERIAL AND METHODS

### Chemicals

Ultra-pure and analytical grade chemicals were supplied from, Merck, Oxoid, and Sigma. DNA markers were purchased from Fermentas. DNA isolation kits were from Promega and Qiagen. Oligonucleotides were synthesized by 1stBase Lab, Malaysia.

### Isolation of 3CP Degrading Bacteria

Soil sample was obtained from paddy field. Bacteria species were isolated using enrichment culture technique. Glycerol stock was prepared using 70% (w/v) glycerol and kept at -80°C for long-term storage.

### Growth Condition

Basal salts and metal salts were prepared in 10x concentration stock solution. Basal salts (10x) contained $K_2HPO_4.{}_3H_2O$ (42.5 g/l), $NaH_2PO_4.H_2O$ (10.0 g/l) and $NH_4SO_4$ (20.0 g/l) whereas, 10x metal salt contained $MgSO_4.{}_7H_2O$ (2.0 g/l), $Fe_2SO_4.{}_7H_2O$ (0.12 g/l), $MnSO_4.4H_2O$ (0.03 g/l), $ZnSO_4.H_2O$ (0.03 g/l) and $CoCl_2.{}_6H_2O$ (0.01 g/l).

The liquid medium for growth contained 1x basal salts and 1x metal salts with 20 mM 3-chloropropionic acid as a carbon source. 3-chloropropionic acid was neutralized with 10 M NaOH to pH 7 and sterilized by filtration. The cell culture was grown in 250 ml flasks containing 100 ml medium at 30°C on a rotary shaker.

### Measurement of Bacterial Growth

Growth curves were plotted by means of measuring the turbidity of the broth medium ($A_{680nm}$) using a spectrophotometer (Jenway 6300). Absorbance readings were taken at 6 hours intervals.

### Determination the Bacterial Morphology

Colony bacteria were observed on 20mM 3CP PJC solid minimal medium.

### Biochemical Test and Staining

Biochemical tests and staining were carried out to determine the bacterial properties.

### Bacteria Identification by 16S Rrna

The genomic DNA was isolated using Wizard® Genomic DNA Purification Kit (Promega). The universal primers were used to amplify the 16S rRNA gene (Table 1). The PCR cycle was set as in Table 2. The PCR product was then purified using QIAquick PCR purification kit (Qiagen). The 16s rRNA gene sequences were extended by designing walking primers as in Table 3.

Table 1: Universal primers used

| Primer | Sequence |
|---|---|
| FD1 | 5′-gag gca gca gtg ggg aat at-3′ |
| Rp1 | 5′-gtg ccc aac tta aat gat gg-3′ |

Table 2: PCR cycles

| Steps | Temperature | Time |
|---|---|---|
| Initial Denaturation | 94 °C | 5 min |
| Denaturation | 94 °C | 1 min |
| Annealing | 55 °C | 1 min |
| Extension | 74 °C | 4 min |
| Final Extension | 74 °C | 10 min |

Table 3: Primers used for sequencing reaction

| Primers Orientation | Primers Name | Primers sequence | GC content |
|---|---|---|---|
| Reverse | Rp1 | 5′-gtg ccc aac tta aat gat gg-3′ | 45.0% |
| | RzRp2 | 5′-gga ctt aac cca aca tct c-3′ | 47.4% |
| | RzRp3 | 5′-cgg tga aag agc ttt aca-3′ | 44.4% |

**Computer Analysis of 16S Rrna Sequence**

BLASTn computer programming from the National Center of Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov) were used. Readable sequences were aligned and compared to those in the nucleotide database. The sequence identities and homology were determined (Wheeler *et al.*, 2000).

## RESULTS

**Bacterial Morphology, Biochemical Tests and Staining Properties.**

The morphology of the single colony was shown in Table 4. Table 5 and 6 showed the staining properties and biochemical test results respectively. From the analysis the bacteria was identified as *Rhodococcus sp.*

Table 4: Cultural characteristics of single colony obtained

| Characteristics | Observation |
|---|---|
| Size of colony | Small |
| Pigmentation | Yellow orange pigment |
| Form (Shape of the colony) | Circular (Unbroken peripheral edge) |
| Margin (Outer edge of colony) | Entire (Sharply defined, even) |
| Elevation | Raised (Slightly elevated) |

Table 5: Staining properties of isolated bacteria

| Staining | Results |
|---|---|
| Gram Staining | Positive |
| Acid Fast Staining | Negative |
| Spore Staining | Negative |

Table 6: Biochemical test results

| Biochemical test | Results |
|---|---|
| Citrate | Negative |
| Indole | Negative |
| Nitrate | Positive |
| Urea | Negative |
| Motility | Negative |
| Methyl Red | Positive |
| Gelatin | Positive |
| Starch | Negative |

**Bacterial Growth in 20mm 3cp Liquid Minimal Medium**

Growth curve of *Rhodococcus sp.* in 20 mM 3CP minimal medium was plotted using semi-logarithmic graph with cell doubling time of 12.50 hours. Growth of the same bacteria was also tested on other halogenated compounds (Table 7).

Table 7: Growth properties of culture in different halogenated compound

| Substrate | Doubling Time |
|---|---|
| 3-chloropropionic acid (3CP) | 12.5 Hours |
| 2-chloropropionic acid (2CP) | No Growth |
| 2,2-dichloropropionic acid(2,2DCP) | No Growth |
| 2,3-dichloropropionic acid (2,3DCP) | No Growth |
| 3-bromopropionic acid (3BP) | 37.6 Hours |
| 2-bromopropionic acid (2BP) | No Growth |

**Bacteria Identification by 16S Rrna**

The 16S rRNA gene sequence obtained was analysed using BLASTn tool**.** The results obtained supported the earlier identification by using biochemical tests. The RNA sequence analysis shared more than 99.6 % identity to the sequences of *Rhodococcus rhodochrous.*

## DISCUSSION

The 3CP degrading bacteria identified as *Rhodococcus sp.* grew well in 20 mM 3CP liquid minimal medium with a doubling time of 12.50 hours. However, growth was 3 times slower in 20 mM 3-bromopropionic acid (3BP). This suggests the chloro substituted compound is a better substrate compared to bromo substituted compound. However, no growth was detected on other halogenated substrate for example 2,2-DCP, 2,3-DCP, 2BP and 2CP. This phenomenon maybe due to the substrates is not subjected to dehalogenase enzyme. Another possibility is these compounds are not uptake into the cell due to the lack of the enzyme uptake system.

Ng (2004) suggested that the percent identity less than 82.45% was not a significant number whereas values higher than 82.45% may be significant to identify *E.coli sp.* In current investigation, 16S rRNA of 3CP degrading bacteria shared more than 99.6% to *Rhodococcus sp*. Therefore, the identity of the bacteria belongs to the *Rhodococcus sp*. In addition to that, data from 16S rRNA analysis was also agreed to the biochemical and staining properties.

*Rhodococcus sp.* is environmentally and biotechnologically important. They are well suited for bioremediation due to their capacity for long term survival in soil, their exceptional ability to degrade hydrophobic and halogenated pollutants even in the presence of more readily assimilable carbon sources (Fournier *et al*, 2002; Maeda *et al.*, 1995; Haroune *et al.*, 2002; Seto *et al.*, 1995). It also accumulates high levels of heavy metals. *Rhodococcus sp.* was also known in degradation of herbicide thiocarbamate (de Schrijver *et al.*, 1997) and herbicide *S*-Ethyl Dipropylthiocarbamate (Shao and Behki, 1995). *Rhodococcus sp.* could have great potential in biodegradation and bioremediation of various xenobiotic including halogenated compounds.

## CONCLUSIONS

A bacterial species had been isolated with cell doubling time of 12.50 and 37.6 hours using 3CP and 3BP as a carbon source in minimal liquid media, respectively. However, no growth was detected in other halogenated compounds which only differ in halogen substitution position. The microorganism was identified as *Rhodococcus sp.* with special ability to degrade pollutants.

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