Asian Journal of Chemistry; Vol. 23, No. 10 (2011), 4255-4259

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

# Trichloroethylene Degradation by Two Phenol Utilizing Strains of *Comamonas testosteroni*

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(*Received*: 30 July 2010; *Accepted*: 3 June 2011) AJC-10021

Environmental isolate *Comamonas testosteroni* RF2 and its derivative strain VM obtained by random chemical mutagenesis were tested for their capacity to degradation of trichloroethylene. Both strains were able to grow on lactate and phenol in the presence of trichloroethylene at concentrations from 0.8-50 mg/L and in the state of resting cells they were able to degrade trichloroethylene during *ca*. 22-24 h. Trichloroethylene degradation by both strains led to more than 95 % mineralization of chlorine. However, VM culture produced significantly higher amount of trichloroacetic acid (3.4 mol %) than maternal strain RF2 (0.15 mol %). Using lactate as the substrate and phenol as the inducer, it was possible to realize three consequent cycles of trichloroethylene degradation by strain RF2 under growing condition, with substantial regeneration of its degradation capacity.

**Key Words: Trichloroethylene, Degradation, Phenol, Bacterial strains.**

## INTRODUCTION

Trichloroethylene (TCE) is one of the most important chlorinated groundwater pollutants in many industrial countries so that the possibility of its microbial degradation is the aim of many research and engineer panels. Some groups of bacteria, possessing mono- or dioxygenases, are able to transform trichloroethylene to labile intermediates consequently undergoing spontaneous hydrolysis yielding various end products and inorganic chlorides[1-4]. However, from the field applicability point of view bacterial cultures differ one from another markedly. Cultivability, transformation capacity, sensitivity to reactive intermediates and spectrum of end products produced are the main factors of choice between available strain(s). Therefore, bacteria utilizing aromatic substrates (especially phenol or toluene) belong to the group of important candidates for field bioremediation, even they differ in their transformation properties[5,6]. As was proved by Futamata *et al.*[7], in phenol growing bacteria, structure of their phenol 2-monooxygenases plays an important role in a level of their affinity to trichloroethylene. Consequently, bacteria showing high affinity to the compound and high transformation rate were found to be members of *Variovorax*, *Burkholderia* or *Comamonas genera*, in the last case especially *Comamonas testosteroni*[8-10]. Despite of slow growth of *Comamonas testosteroni* on phenol at its higher concentrations the species is well known as excellent degrader of vast array of aromatic substrates. Its capacity to degrade toluene and ethylbenzene[11], 2-, 3- and 4-chlorphenols[12,13], 4-sulphophenol[14], nitrophenol and nitrobenzene[15], *p*-toluenesulfonate[16], biphenyl and some PCB congeners[17-19] 3-chloroaniline and 3,4-dichloroaniline[20,21] have been already described.

In previous work[22] we isolated phenol utilizing strain RF2 showing relatively high trichloroethylene transformation capacity at initial trichloroethylene concentration about 1 mg/L. However, in further tests the strain in the state of resting cells showed low transformation capacity when initial trichloroethylene concentrations of about 10 mg/L were used (not published). Therefore, random mutagenesis of the strain RF2 was performed, aimed to obtaining a strain able to degradation of elevated trichloroethylene concentrations. The process yielded strain VM able to grow on increased phenol concentration.

In this work we studied trichloroethylene degradation by these two strains aimed to their potential use for bioremediation of groundwater contaminated by a pollutant mixture including trichloroethylene.

## EXPERIMENTAL

**Strains description:** *Comamonas testosteroni* RF2 was isolated from activated sludge fed by phenol[22] and identified on the base of its growth and biochemical properties and fatty acids profile (Similarity Index 0.855). The strain is able to grow on phenol at concentrations up to 300 mg/L. It can utilize

some organic salts (acetate, citrate, lactate, glyoxylate). It is deposited in Czech Collection of Microorganisms under the catalogue number CCM 7350. *Comamonas testosteroni* VM was derived from RF2 strain by mutagenesis with help of sodium azide (1 mg/L, 3 h) and obtained by selection on agar plates with 500 mg/L phenol as the only carbon source. Besides to its capability to grow at elevated phenol concentrations it differs from the maternal strain by its inability to utilise citrate.

**Trichloroethylene degradation tests:** All the tests were made in triplicate or quadruple; abiotic controls were always done.

**Trichloroethylene degradation by the resting cells:** Cells were grown on citrate or lactate (500 mg/L) and phenol (200 mg/L) for 22 h and then 200 mg/L of phenol were added. After 2 h the cells were harvested by centrifugation (10,000 G, 10 min) and washed twice by phosphate buffer pH 7.2. Cells were suspended in mineral medium[22] and density was adjusted to OD620 = 0.05-0.1. 10 mL of inoculums was placed into 40 mL glass vials (Supelco, USA) enclosed with Teflon-coated silicon septa. Immediately before closure, determined volumes of trichloroethylene stock water solution (1.2 g/L) were dosed into the sample vials by means of gas-tight syringe (Hamilton, USA) to set an initial trichloroethylene concentration of 1.0 mg/L in the aqueous phase. Prepared vials were placed on rotary shaker (80 rpm, 25 ºC) for 24-144 h including several blank vials without inoculum.

**Trichloroethylene degradation under growing condition:** 10 mL of mineral medium with lactate (100 mg/L), phenol (100 mg/L) and yeast extract (20 mg/L) was pipetted into 40 mL sample vials. Each sample vial was inoculated with 10 mL of cell suspension and immediately after addition of trichloroethylene (final concentration ranged from 0.8-50 mg/L) the vials were closed. Trichloroethylene stock water solution or methanol solution (60 g/L) were used. Cultivation was done statically in dark, at 25 ºC for 5 days and final trichloroethylene concentrations were then determined by gas chromatography.

**Repeated trichloroethylene degradation under growing condition:** Three sets of sample vials containing 10 mL of mineral medium with lactate, phenol and yeast extract at above mentioned concentrations were prepared. All were inoculated with 10 mL of cell suspension *C. testosteroni* RF2. Trichloroethylene at concentration of about 11 mg/L was added to all sets of vials. These test sets were incubated 5 days on rotary shaker (80 rpm) at 25 °C and then final trichloroethylene concentrations in the first set of vials were measured. The remaining trichloroethylene in the second and third test sets were subsequently stripped by air and lactate, phenol and yeast extract as well as trichloroethylene were added again at the starting concentrations. Cells surviving the first degradation formed the inoculum. Cultivation of second and third sets was carried out in identical manner and after it trichloroethylene final concentrations in vials of second set were determined by gas chromatography. The remaining trichloroethylene in the third test set were removed again, substrates as well as trichloroethylene were added and the vials were incubated as mentioned above.

Further, another three sets of sample vials were prepared and followed in parallel with previous experiment, where trichloroethylene was not added during the first or first and second stages of degradation, but was only added in the second or third stage of degradation, respectively (control sets).

**Cell number estimation:** Plate count method after decimal dilution of suspensions was used at the end of each degradation phase. Bacteria were cultivated on tryptone yeast extract agar (tryptone 6 g, yeast extract 3 g and agar 15 g/L) at 25 °C for 2 days.

**Determination of trichloroethylene concentration in liquid phase:** Trichloroethylene concentrations were determined by gas chromatography on a Hewlett-Packard 5890 gas chromatograph after sample concentration on Purge and Trap (P and T) system Tekmar LSC 2000 (sorbent Vocarb 4000 from Supelco; desorption temperature 250 °C). Volatile substances were separated on capillary column Quadrex (methylphenyl cyanopropyl Silicone 30 m × 0.53 mm ID, 3 mm film, from Quadrex Corporation, USA) and subsequently detected in flame-ionisation detector. 5 mL of sample were dosed into the concentrator either directly the culture (with trichloroethylene concentrations up to 1 mg/L) or after defined dilution of samples containing higher trichloroethylene concentrations. If not explicitly stated, text as well as tables always shows trichloroethylene concentrations in the water phase.

**Determination of chloride ions released during degradation:** Enriched and induced cells were washed and suspended in a chloride-free medium and trichloroethylene degradation by resting cells was performed. The quantity of chloride ions was determined by the spectrophotometric method according to Iwasaki[23] and corrected for the blank tests (cells without trichloroethylene and trichloroethylene in distilled water). The quantity of chloride ions released during degradation was related to the quantity of chlorine ions contained in removed trichloroethylene. In order to calculate trichloroethylene concentrations in the gaseous phase of vials the dimensionless Henry constant for trichloroethylene at 25 ºC, H = 0.4, was used[24].

**Determination of trichloro acetic acid (TCA):** Validated method of LABTECH Brno Company was used. Trichloroethylene degradation by both strains in the state of resting cells were done and the suspensions were centrifuged (10,000 G, 10 min) for cell removal. Obtained samples were concentrated and trichloroacetic acid was converted to methyl ester by methanol. Quantity of methylester was determined by gas chromatography with electron capture detector, on the base of a calibration curve. The trichloroacetic acid levels produced during degradations were compared with quantities of trichloroethylene removed.

## RESULTS AND DISCUSSION

**Time ability of *Comamonas testosteroni* RF2 resting cells to trichloroethylene degradation:** Owing to the slow growth of the RF2 strain at higher phenol concentrations, sodium citrate was used for its rapid multiplication in the blend with 200 mg/L of phenol, with the additional induction by phenol 200 mg/L. The time course of trichloroethylene degradation by resting cells was than determined and results are graphically presented in Fig. 1.

The ability of *C. testosteroni* RF2 in the form of resting cells to degrade trichloroethylene was limited with time to *ca.* 22-24 h. This could be considered as sufficient time for a

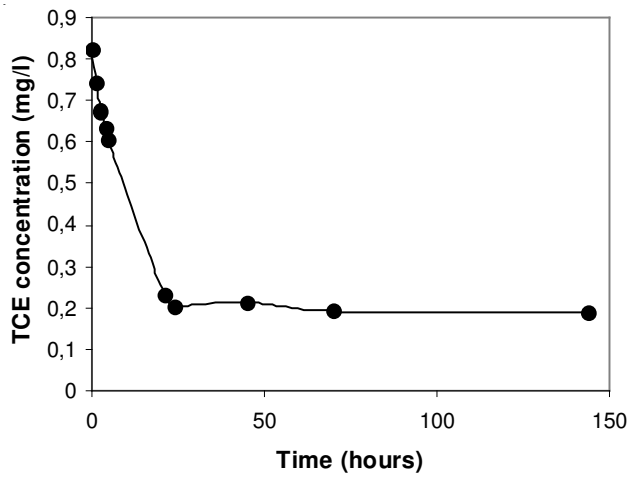


Fig. 1. Course of trichloroethylene degradation by resting cells of *C. testosteroni* RF2

potential application. As it was proved later, the time course of trichloroethylene degradation by VM strain showed similar pattern (data not shown) suggesting that both strains should be repeatedly induced for possible longer degradation process.

**Trichloroethylene degradations under growing conditions:** Cell multiplying in the presence of trichloroethylene is important prerequisite for use of bacteria in contaminated groundwater, so the capabilities of RF2 strain and its derivative VM to grow on the mixture of phenol and lactate at different trichloroethylene concentrations (0.8-50 mg/L) were tested. Levels of trichloroethylene degradation were after 5-day cultivations measured too and the results are summarized in Table-1.

TABLE-1
TRICHLOROETHYLENE DEGRADATION BY RF2 AND VM STRAINS AFTER THEIR GROWTH ON LACTATE AND PHENOL IN PRESENCE OF TRICHLOROETHYLENE (MEANS AND SD, FOR n = 3)

| Initial TCE conc. (mg/L) | Culture growth | | TCE removal (%) | |
|---|---|---|---|---|
| | RF2 | VM | RF2 | VM |
| 0.76 ± 0.02 | ++ | ++ | 100 | 100 |
| 1.51 ± 0.03 | ++ | ++ | 100 | 100 |
| 5.05 ± 0.14 | ++ | ++ | 54.6 ± 2.2 | 73.8 ± 1.6 |
| 10.0 ± 0.26 | ++ | ++ | 28.7 ± 1.4 | 39.7 ± 2.1 |
| 25.2 ± 1.1 | ++ | ++ | N | 10.0 ± 0.8 |
| 49.8 ± 1.7 | + | ++ | N | 0 |

+: Growth on lactate and phenol in 72 h, ++: growth on lactate and phenol in 24 h, N: not tested.

Tests showed the ability of both cultures to grow in the presence of trichloroethylene, though the growth of RF2 at 50 mg trichloroethylene/L was slightly slower. Presence of phenol, lactate (both at 100 mg/L) and yeast extract (20 mg/L) led to complete trichloroethylene removal at its concentrations 0.8 and 1.5 mg/L and to partial trichloroethylene removal at 5 and 10 mg/L. At these two last cases the levels of trichloroethylene degradation by VM strain exceeded values obtained with RF2 strain, so VM appeared to be more suitable for degradation of higher trichloroethylene concentrations. On the other hand, not even this bacterium was able to remove the highest trichloroethylene concentrations (50 mg/L) owing to the low cell density grown on applied concentrations of substrates.

**Chlorides production and trichloroacetic acid formation:** Because of the great importance of end products formed by bacterial during trichloroethylene degradation, levels of inorganic chlorides and trichloroacetic acid produced by the process were determined. Tests were performed separately and summary of the results is given in the Table-2.

TABLE-2
FORMATION OF TRICHLORO ACETIC ACID (TCA) AND INORGANIC CHLORIDES AFTER TCE DEGRADATION BY BOTH STRAINS

| Chlorides production (mol %) | | Trichloro acetic acid formation (mol % of trichloroethylene degraded) | |
|---|---|---|---|
| RF2 | VM | RF2 | VM |
| 97.8 | 95.3 | 0.15 | 3.4 |

Trichloroacetic acid is the most important potential end product of trichloroethylene degradation. Its formation was proved in the case of trichloroethylene transformation by *Methylocystis* sp. strain M3 but not in the cases of trichloroethylene degradation by purified toluene mono- and dioxygenase[2,25].

Surprisingly, we found different levels of TCA formation using strains of *C. testosteroni*. Trichloroethylene degradation by RF2 isolate, characterised by high extent of chlorine mineralization and low level of produced trichloroacetic acid, appeared to be environmentally more acceptable than the process effectuated by strain VM. Formation of trichloro acetic acid during trichloroethylene degradation by bacteria growing on phenol (or by purified phenol 2-monooxygenase) has not been described yet. Ishida and Nakamura[4] have detected no formation of tri- and dichloro acetic acids by *Ralstonia* sp. KN1-10A.

Trichloro acetic acid production by strain RF2 may be considered as nearly negligible. In addition to this, the acid was proved as naturally originating compound in some soils. Hoekstra *et al.*[26], found its concentrations from 0.2-4.6 µg/kg soil dry mass, Plumacher[27] from 1.4-120 µg/kg soil dry mass and Frank[28] even up 380 µg/kg.

Generally, from the end products point of view, trichloroethylene degradation performed by environmental strain *C. testosteroni* RF2 may be considered as the acceptable process for field applications.

**Repeated trichloroethylene degradation by RF2 under growing conditions:** A short-time activity of cometabolic degradation is a common feature of natural cultures caused by regulation mechanisms of bacterial cells. This unwilling phenomenon may be suppressed by the use of genetically modified bacteria with constitutive production of the key enzyme, but their use usually requires a certain legal certification process. An alternative way how to prolong the time of degradation may be cyclic degradation during which regeneration of the transformation capacity of a natural culture takes place. Based on the above mentioned results we tested the ability of strain RF2 to perform three consecutive trichloroethylene degradations under growing condition, each at initial trichloroethylene concentration above 10 mg/L. Besides to the determination of trichloroethylene removal the cell counts at the end of each stage of degradation were estimated. Results of the three test sets and three control sets are given in the Table-3.

TABLE-3
REPEATED DEGRADATION OF TCE BY STRAIN RF2 UNDER GROWING CONDITION (MEANS AND SD, FOR n = 4)

| Stage No. | Set of vials | Cell number (CFU/mL) | TCE concentration (mg/L) | | TCE removal (%) |
|---|---|---|---|---|---|
| | | | Initial | Final | |
| 1 | Test | $2 \times 10^6$ | 11.03 ± 0.09 | 2.11 ± 0.20 | 80.8 |
| | Control | $2 \times 10^7$ | 0.0 | 0.0 | – |
| 2 | Test | $6 \times 10^6$ | 11.03 ± 0.09 | 3.30 ± 0.19 | 70.0 |
| | Control | $8 \times 10^7$ | 11.03 ± 0.09 | 1.28 ± 0.16 | 88.3 |
| 3 | Test | $6 \times 10^6$ | 11.03 ± 0.09 | 4.37 ± 0.34 | 60.3 |
| | Control | $9 \times 10^7$ | 11.03 ± 0.09 | 1.16 ± 0.22 | 89.5 |

Whole test essentially showed the possibility of repeating growth of the investigated strain in presence of trichloroethylene followed by pollutant degradation. As generally expected, the most significant level of trichloroethylene removal was found in the first stage; in the following ones mild reduction in cell degradation capacity already occurred in test sets (80.8-70.0-60.3 % degradation), nevertheless removal of about two thirds of added trichloroethylene in the second and third stages may be regarded as a promising result. Therefore, used procedure enabled to attain substantial regeneration of the culture's degradation capacity by repeating additions of growth and induction substrates as well as oxygen. However, data obtained in control sets (88.3-89.5 % degradation in second and third stage, respectively) and higher cell counts in all control stages showed that the mildly reduced extents of trichloroethylene degradation in second and third test sets did not originate from cell population ageing or accumulation of metabolic waste products, but they were provoked by trichloroethylene degradation itself, *i.e.*, by slight toxicity of trichloroethylene, reactive intermediates (for example trichloroethylene-epoxide) and/or degradation products. This fact was supported by another phenomenon connected with trichloroethylene degradation. Cell suspensions of the first stage, both from test and control vials, were examined microscopically before plate counting. Cells of control set (no trichloroethylene present) were organized mostly in a form of microflocs (Fig. 2), whereas cells of test set after trichloroethylene degradation formed fine suspension of free cells (Fig. 3). This effect might indicate that trichloroethylene and/or its degradation intermediates/products not only decreased numbers of cells remaining alive in test suspensions but also they provoked some change(s) on cells surface and changed their aggregation properties.

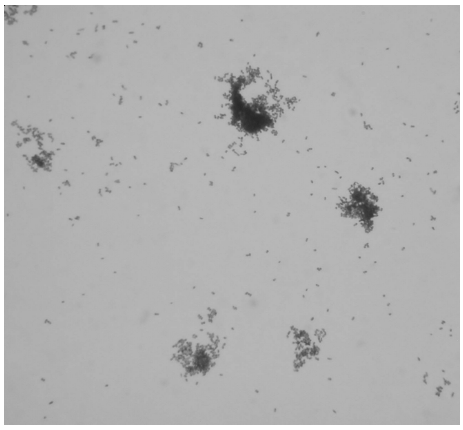

Fig. 2. Microflocs formation by RF2 cells without trichloroethylene degradation (stained by crystalviolet, magn. 1000x)

First stage of trichloroethylene degradation even showed another interesting phenomenon concerning extent of trichloroethylene removal. 80.8 % degradation found in this test was much higher than the result obtained in preliminary testing (28.7 %, Table-1). The difference may be explained by cultivation character of appropriate cultures; whilst in preliminary testing the vials were incubated statically, in the experiment of repeated trichloroethylene degradation a mild movement on rotary shaker was used. This result showed that favourable conditions for the strain are crucial for its strong transformation capability and successful process of remediation.

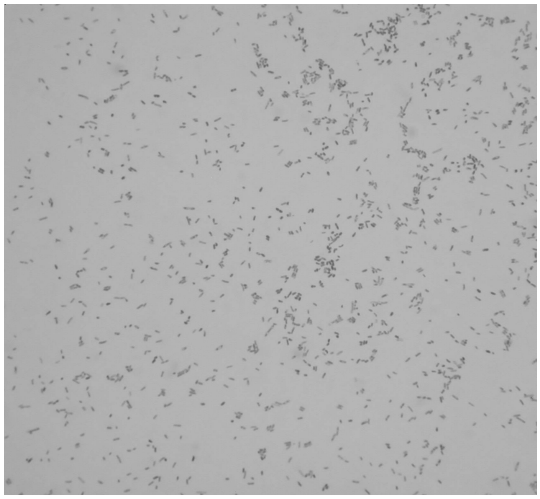

Fig. 3. Fine RF2 cell suspension after trichloroethylene degradation (stained by crystalviolet, magn. 1000x)

## Conclusion

Present results showed that it is relatively simple to obtain bacterial strain able to degradation of elevated trichloroethylene concentrations. However, it was also proved that higher extent of pollutant degradation is not only the indicator of strain's pertinence for real application and that formation of end products must be carefully taken into account too. From this point of view, natural strain *Comamonas testosteroni* RF2 should be preferred for field bioremediation because of its significantly lower production of trichloro acetic acid as the end product of trichloroethylene degradation and higher level of chlorine mineralization. In the case of trichloroethylene pollution at concentrations of about 10 mg/L or slightly higher the trichloroethylene elimination process could be realized by the strain under growing condition, in two or three degradation stages in which the degrader is affected by own trichloroethylene degradation at low extent only.

From the general point of view, the work proved that formation of trichloro acetic acid as the end production of trichloroethylene degradation is not limited to processes carried out by some methylotrophic bacteria and that the compound may be produced at low concentration when degradation is done by some bacteria possessing another type of oxygenase.

## ACKNOWLEDGEMENTS

The authors thank LABTECH Brno Company for TCA determinations. The work was partially supported by the Ministry of Education, Youth and Sports of the Czech Republic, project No. MSM 7088352101. This article was also created with support of Operational Programme Research and Development for Innovations co-funded by the European Regional Development Fund (ERDF) and National Budget of Czech Republic within the framework of the Centre of Polymer Systems Project (Reg. No. CZ.1.05/2.1.00/03.0111

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