The effects of culture condition on the degradation of PCE and production of bio-surfactant by *Pseudomonas aeruginosa*

**Jebakumar Solomon** RD 1,*, Raymond L Legge 2

1 Dept. of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, India
2 Dept. of Chemical Engineering, University of Waterloo, Waterloo, Canada


Abstract
Perchloroethylene (PCE) is an environmental pollutant and water contaminant. PCE biodegradation by *Pseudomonas aeruginosa* ATCC 9027 occurred efficiently when PCE was supplied as a sole carbon source rather than with the co-substrate glucose. Fifty percent degradation of PCE was observed in 6.3 hr when PCE was the sole carbon source and 90 hr with glucose and PCE. The degradation intermediates, trichloroethylene and dichloroethylene, were detected as quantifiable amounts and vinyl chloride and ethylene were observed in trace amounts. When PCE was used as the sole carbon source, rhamnolipid production was higher, which enhanced the degradation of PCE likely by reduction in surface tension and making it more readily available for degradation by *P. aeruginosa*.

Keywords: Perchloroethylene, Rhamnolipid, Biosurfactant, Bioremediation, *Pseudomonas aeruginosa*.

INTRODUCTION

Tetrachloroethylene (PCE) is used as an industrial degreasing solvent and fumigant and is frequently detected as a volatile organic compound in municipal ground water supplies (Carter and Jewell, 1993). Like many chlorinated hydrocarbons, PCE is a central nervous system depressant which causes ill effects on human health like skin irritation, dizziness, headache, sleepiness, confusion, nausea, unconsciousness, and death (Patel et al., 1977). Bioremediation of industrial toxic pollutants will provide a pollution control and waste minimization technology to maintain the environmental health. Bioremediation of PCE by microorganisms is an attractive remediation technology to overcome the environmental pollution and it is an economic route for remediation of chemically contaminated sites. This can be achieved by inoculation with known strains capable of degrading specific compounds (Juan et al., 2000).

Biosurfactants are amphipathic molecules produced by a wide variety of bacteria, yeast and filamentous fungi (Mercade and Manresa, 1994) which can enhance bioremediation processes by increasing the bioavailability of recalcitrant xenobiotics both for in-situ bioremediation and ex-situ slurry-reactor bioremediation. Rhamnolipids are extra cellular glycolipid biosurfactants produced by several strains of *Pseudomonas* spp., growing on diverse carbon source long chain hydrocarbons, carbohydrates, glycerol and vegetable oils (Linhardt et al., 1989; Bodour et al., 2003) which cause the cell surface of *Pseudomonas* spp. to become hydrophobic through release of lipopolysaccharide (LPS). Rhamnolipids are better than their synthetic counter parts in enhancing the solubility of alkanes, polycyclic aromatic hydrocarbons (PAH), aromatics and polychlorinated biphenyls (Scheibenbogen et al., 1994; Kanga et al., 1997). The form of carbon source can influence biosurfactant production, for example, vegetable oils can promote rhamnolipid production by some *Pseudomonas aeruginosa* strains (Matta-Sandoval et al., 2000).

When compared to other chemical contaminants, tetrachloroethyene (PCE) is a recalcitrant compound to degrade biologically and some of the microorganisms in the nature have the mineralization and degradation potential of PCE. The present study is aimed at analyzing the effects of different culture conditions on the biodegradation of PCE and production of rhamnolipid by *P. aeruginosa* ATCC 9027.
MATERIALS AND METHODS

Culture conditions for Pseudomonas aeruginosa ATCC 9027

Pseudomonas aeruginosa ATCC 9027 was purchased from the American Type Culture Collection (Rockville, Maryland, USA) was maintained in the slant culture with a medium containing 2% peptone, 1% glycerol, 1% K$_2$SO$_4$, 0.14 % MgCl$_2$, agar 1.5%, at a pH of 7.2. The bacterial growth over time was analyzed in Kay’s minimal medium consisting of 0.3% NH$_4$H$_2$PO$_4$, 0.2% K$_2$HPO$_4$, 0.2% glucose, 0.5 mg/liter FeSO$_4$, 0.1% MgSO$_4$ (Siegmund and Wagner, 1991). The modified peptone glucose ammonium salt (PGAS) medium with 0.02 M NH$_4$Cl, 0.02 M KCl, 0.12 M Tris-HCl, 0.0016 M MgSO$_4$, 1% Peptone, 0.5% glucose, pH 7.2 (Zhang and Miller, 1992) was used for the production of rhamnolipid by P. aeruginosa ATCC 9027. A series of positive (PCE + glucose in PGAS medium), negative control (PCE + no glucose in PGAS medium) and test experiments were performed to confirm the biodegradation of PCE both in presence and absence of glucose in PGAS medium supplemented with PCE. One ml of P. aeruginosa preculture from Kay’s medium was inoculated into 100 ml of PGAS medium in seven sets of 160 ml serum bottles sealed with screw caps and Teflon coated butyl rubber septa and incubated at 37°C. PCE was supplemented in the medium at 12.9 μg/ml which is slightly higher than the sub lethal concentration to P. aeruginosa. The biomass of the cells was calculated by measuring the dry weight of the cells following filtration through 0.2 μm Whatman nitrocellulose filter paper.

Analysis of PCE degradation by gas-liquid chromatography (GLC)

PCE biodegradation by P. aeruginosa was analyzed by quantifying the amount of residual PCE in the culture broth at different time intervals at 1, 12, 24, 36, 48, 72, 90 and 120 h. The residual PCE from the culture broth was extracted with methyl tertiary butyl ether by EPA Method 502.1. Five ml of culture broth was added to 50 μl of 10 mg/ml decafluorobiphenyl acetone, 1 ml of methyl tertiary butyl ether, 2g Na$_2$SO$_4$ mixed vigorously for 4 min and allowed to separate for 20 min. The upper organic phase was used for analysis of the residual PCE and PCE degradation was determined from the mean value and standard deviation of three replicates. To identify the PCE degradation intermediates in batch experiments, trichloroethene (TCE), and cis-dichloroethene (cis-DCE) were quantified by organic solvent extraction of the medium followed by spiking in sample prior to the GLC analysis. The gas phase intermediates; vinyl chloride and ethene were analysed by injection of headspace samples into an Agilent 6890 gas chromatograph equipped with HP-1 megabore column (0.32 mm, 30 m length, 0.25 μm) with flame ionization detection (FID). The chromatograph was operated at 60°C for 5 min then ramped to 200°C at 20°C per min. The injection port was maintained at 250°C and the detector at 290°C with helium as a carrier gas at a flow of 10 ml/min. The GC calibration standards were prepared with a PCE solution of 1 mg/ml from Sigma Aldrich. PCE concentration was expressed as mean and standard deviations based on the results obtained from triplicate samples.

Assay of rhamnolipid production and surface tension determination

A standard solution of rhamnose monohydrate was prepared in a working concentration of 0.05 mg/ml. One ml of the standard solution was mixed with 4.5 ml of solution A - deionized water/H$_2$SO$_4$ (1:6) and kept in ice, mixed well by vortexing, heated in boiling water bath for 10 min and allowed to cool. The 0.1 ml of reagent B - mercaptoacetic acid/water (1:30) was added, kept in dark for 3 h and the absorbance values were measured at 400 and 430 nm with deionized water as a blank (Scherz and Bonn, 1998). Rhamnolipids were extracted from the culture supernatant by centrifugation at 7,000 rpm. The supernatant was acidified with 10 M hydrochloric acid to pH 2.0, and the precipitated rhamnolipids were centrifuged at 12,000 rpm, pellets were dissolved in chloroform/ethanol (2:1) mixture, solvent mixtures were evaporated and the pure rhamnolipid concentrations were analyzed using a scanning spectrophotometer. Steady-state interfacial tension between the rhamnolipid and PCE was determined by the drop volume method (Miller and Fainerman, 1998; Clifford et al., 2007) using the capillary tip connected with motor driven syringe. Twenty ml of rhamnolipid solution prepared in 10 mM phosphate buffer (pH 7) at 0-175 mg/ml was equilibrated with 20 μl of PCE and 3 ml of the equilibrated rhamnolipid in a glass cuvette and PCE was dispensed at a flow rate of ml/h by capillary tip and the assays were performed in triplicates.

RESULTS

The ability of P. aeruginosa to degrade PCE was tested in presence of glucose and PCE as a major carbon source and active degradation was confirmed by both the disappearance of PCE and the generation of subsequent degradation metabolites. Pseudomonas aeruginosa ATCC 9027 showed pronounced differences in biodegradation ability of PCE when different carbon sources were supplemented in the medium. Following 1h of incubation, the PCE concentration was found to be 11.52 ± 1.15 μg/ml. After 12h, the amount of PCE was reduced to 9.71 ±
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0.87 μg/ml. Further degradation of the PCE was observed as 8.45 ± 0.62 at 24h and 4.61 ± 1.18 μg/ml at 120h. The degradation potential was observed as 15.7% and 60.0% respectively at 12 and 120 h of incubation of bacteria with PCE and glucose. Fig. 1 presents the degradation pattern of PCE by *P. aeruginosa* ATCC 9027 under different conditions. In another set of experiments, PCE biodegradation was checked with PCE as a carbon source without glucose. The residual amount of PCE in the medium was checked with PCE as a carbon source without glucose.

Another set of experiments, PCE biodegradation was monitored with *P. aeruginosa* ATCC 9027 in absence of co-substrate and with glucose. Fig. 1 shows the enhanced degradation of PCE by *P. aeruginosa* ATCC 9027. Metabolites were quantified and expressed as mean ± standard deviation. Deviations were calculated as total analytical error (5%) of triplicate analysis of experiment.

In a control experiment, the loss of PCE was not observed. The medium containing glucose and PCE as a carbon source inoculated with *P. aeruginosa* at 90th h the 50% biodegradation of PCE was observed and in another experiment in which the degradation of PCE was monitored with *P. aeruginosa* in the medium supplemented with PCE as a sole carbon source, the 50% biodegradation ability was observed at 6.3h of incubation. This demonstrates that the rate of degradation of PCE is enhanced by the *P. aeruginosa* and the carbon sources play a vital role in biodegradation ability of PCE.

The degradation metabolites; TCE and DCE were simultaneously quantified based on the peak area of gas chromatogram. Table 1 provides a comprehensive picture of the enhanced degradation of PCE by *P. aeruginosa* ATCC 9027 in absence of co-substrate and their mass balance calculations of degradation products of PCE. The degradation of PCE (initial quantity at 1 h: 11.52 ± 1.15 μg/ml) to its intermediates, trichloroethylene (TCE) and dichloroethylene (DCE) was observed as 1.25±0.15 and 2.01±0.49 μg/ml at 120h of incubation in PCE containing medium. The degradation potential was observed as 50% biodegradation ability was observed at 6.3h of incubation which represent the degradation potential of 91.3 and 98.2% respectively.

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- **Figure 1:** Degradation of PCE by *P. aeruginosa* ATCC 9027 at different experimental conditions (i) PCE as the sole source of carbon in which 50% degradation of PCE was observed at 6.3 h (ii) PCE with glucose as the co-substrate, at 90th h 50% of PCE was degraded.

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- **Table 1:** Mass balance determined over the degradation of tetrachloroethylene (PCE) into trichloroethylene (TCE) and dichloroethylene (DCE) by *P. aeruginosa* ATCC 9027. Metabolites were quantified and expressed as mean ± standard deviation. Deviations were calculated as total analytical error (5%) of triplicate analysis of experiment.

The growth and biomass production for *P. aeruginosa* were also monitored with the PCE biodegradation analysis. The bacterial cell mass was highest in experiments based on PCE as a sole carbon source rather than glucose. Under such condition the biodegradation rate of PCE was higher, suggesting that the increased biomass of *P. aeruginosa* and bioavailability of PCE was relatively high due the substantial quantity of rhamnolipid production. The rhamnolipid production ability were monitored at frequent intervals. After the 30 h of incubation, the rhamnolipid production was higher and appeared to be ceased and attained the constant level. The concentration of rhamnolipid was directly proportional to the pigmentation of the strain in the spent culture medium and the pigment production was observed by vinyl chloride and ethylene were detected in trace amounts.
The formation of blue colour from the 10h of incubation in the culture medium.

The quantity of rhamnolipid produced by the *P. aeruginosa* was analyzed at different intervals and secretion of rhamnolipid appeared after 12h of growth and continued up to 120h. Amount of rhamnolipid produced in PCE in absence of glucose was observed as 0.061 ± 0.12 to 0.642 ± 0.101g/l from 1 to 120h of incubation. The results manifested in that incubation with Kay’s minimal medium with PCE as a sole carbon source maintained the constant and higher production of rhamnolipid from 12 to 72h beyond that there was a decrease in the rhamnolipid level in the spent culture medium (Fig. 2). Incubations of PCE with glucose in mineral salts medium showed 0.040 ± 1.23 to 0.189 ± 0.94 g/l from 1 to 120 h of incubation. The quantity of rhamnolipid secreted from 12 to 36h was fairly uniform and from 48h onwards there was a slight increase in synthesis of biosurfactant found in the spent culture medium.

The biosurfactant produced by *P. aeruginosa* ATCC 9027 strain reduced the surface tension of the growth medium from 52.4 ± 0.23 mN/m to 12.4 ± 0.41 mN/m (mean ± SD, n = 3), at the 48h of growth in PCE medium, demonstrating biosurfactant synthesized by *P. aeruginosa* was efficient in reducing the surface tension of growth medium. The PCE solubility was significantly higher than its solubility in the absence of rhamnolipid and increased linearly according to the biosurfactant concentration.

**DISCUSSION**

Microbial communities can dechlorinate tetrachloroethene (PCE) and trichloroethene (TCE) to dichloroethene (DCE), vinyl chloride (VC), and finally to ethene (Maymo-Gatell et al. 1999; Holmes et al. 2006). According to earlier reports, PCE is a recalcitrant contaminant and non-biodegradable by aerobic microorganisms (Maymo-Gatell et al., 1997). However the present study showed that the PCE can be metabolized by an aerobic microorganism and is supported by previous reports (Ryoo et al., 2000; Ryoo et al., 2001; Shim et al., 2001; Marco-Urrea et al., 2008). The aerobic biodegradation by *P. aeruginosa* is mediated by enzymes that can degrade mixtures of all chlorinated ethenes including PCE. *P. aeruginosa* can thus play a role in detoxification of PCE. The multienzyme complex, tolueo-xylene-monoxygenase (ToMO) in *P. stutzeri* OX1 can aerobically degrade PCE and TCE. ToMo enzyme complex consist of hydroxylase with a catalytic oxygen-bridged dinuclear center encoded by *touABE*, NADH-ferredoxin oxidoreductase (*touF*), mediating protein (from *touD*), and the Rieske-type ferredoxin encoded by *touC* (Bertoni et al., 1998; Chauhan et al., 1998; Ryoo et al., 2000; Shim and Wood, 2000). At 24h, 85% of PCE was degraded by *P. stutzeri* OX1 (Ryoo et al., 2000) and it was 91% by *P. aeruginosa* ATCC 9027. Previous reports suggest that the PCE degrading bacteria *P. aeruginosa* strain PAO1, *P. stutzeri* OX1, *P. putida* and *P. syringae* have a chemotactic response towards PCE and TCE (Parales et al., 2000; Varder et al., 2005; Kim et al., 2006) and *P. aeruginosa* strain PAO1 formed dense zones around agarose plugs containing 1 mM ethylene (Kim et al., 2007). This kind of migration potential and solubilization of PCE by rhamnolipid might be used to enhance biodegradation processes involving chlorinated ethylene.

The growth of bacteria and the rhamnolipid production were directly proportional to one another and the biosurfactant production was found to be higher when the bacterial growth and cell mass attained stationary phase. The rhamnolipid production at stationary phase is also described by Ron and Rosenberg et al. (2001). The optimum level of rhamnolipid production can be achieved when the water soluble carbon source, glucose is used in the growth medium of *P. aeruginosa* (Linhardt et al., 1988). However the present study results in the conclusion that the PCE biodegradation and rhamnolipid production was best achieved in Kays mineral medium supplemented with PCE as the sole carbon source with *P. aeruginosa* ATCC 9027 and rhamnolipid production was enhanced by the availability of PCE as the only carbon source in the minimal medium. Rhamnolipid produced by *P. aeruginosa* ATCC 9027 has the potential for use in surfactant-enhanced aquifer remediation (SEAR) processes of PCE, the solubility of PCE is increased linearly by the biosurfactant rhamnolipid. Clifford et al., (2007) reported that change in interfacial tension from 47 mN/m to 10 mN/m and which was consistent with the present report and the PCE dechlorination was fastidious with the production of rhamnolipid.

The limiting factor in biodegradation of xenobiotics is the limited availability to biodegrading microorganisms by increased sorption of organic molecules themselves (Van Delden et al., 1998). Biosurfactants like rhamnolipids can enhance the degradation ability of PCE by desorbing the bound substrate molecules of PCE and making them more readily available to the microorganisms.

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