Wat. Sci. Tech. Vol. 22, No. 9, pp. 59–63, 1990.
Printed in Great Britain.

0273–1223/90 $0·00 + ·50

REMOVAL OF COLIPHAGES FROM WASTEWATER EFFLUENT BY PHOTOTROPHIC BACTERIA

H. Hirotani*, Y. Agui, M. Kobayashi and E. Takahashi

College of Agriculture, Kyoto University, Kyoto, 606, Japan
**Present Address: College of Agriculture, Ehime University, Matsuyama, 790, Japan*

ABSTRACT

A wastewater purifying process using a phototrophic bacterium, Rhodopseudomonas capsulata, is presently operating in several countries. Removal of coliphage during the process was assessed by a field-test and a model study. It was found that 97.4% of coliphage was removed during the purification of wastewater from a pigpen. The model study was performed to ensure that the removal was due to biomass of the phototrophic bacterium, which produces an antiviral substance. Phage inactivation by chloroform-methanol extract from the bacterium with the presence of kaolinite as a contaminating particulate is also shown to describe the efficiency of the antiviral substance from the phototrophic bacterium to wastewater.

KEYWORDS

Phototrophic bacteria; removal of coliphage; wastewater effluent; porcine feces; antiviral substance.

INTRODUCTION

Rhodopseudomonas capsulata is a purple nonsulfur bacterium widely distributed in the environment, playing an important role in the preservation of water in its natural habitat (Kobayashi and Tchan, 1973). The bacterium is utilized in wastewater management sites (Kobayashi et al., 1978) which are operating in several countries including Brazil and Japan. Another feature of this bacterium is the production of an antiviral substance within its cell, which inactivates pathogenic viruses in vitro without any toxicity to the host cells (Kobayashi and Hirotani, 1987a).

The purpose of this study was to investigate if growing R. capsulata potentially removes viruses in the effluent at wastewater treatment sites. It was shown from a model experiment that 90% of the coliphage in wastewater was removed during the wastewater purifying process using this bacteria. This agrees with a field study also presented in this report.

MATERIALS AND METHODS

Bacteria and phage

Escherichia coli strain B was used as the host for coliphages throughout the study. They were grown in F medium (pH 6.8) which contains 1.0 g NH_4Cl, 0.1 g $MgSO_4$, 1.5 g KH_2PO_4, 3.5 g Na_2HPO_4, and 9.0 g lactate per litre of distilled water (Adams, 1950).

R. capsulata cells originally isolated and maintained in our laboratory were grown under

anaerobic conditions at an ambient temperature with constant illumination at 3000 lux. Cultures were routinely grown in 80-litre acrylic vessel containing 50 litres of medium as follows: 0.8 g KH_2PO_4, 0.2 g $MgSO_4$, 0.1 g NaCl, 0.05 g $CaCl_2$, 5g Na-propionate, 0.1 g yeast extract, and 0.5 g NH_4Cl per litre (pH 6.8). Forty litres of the culture solution was withdrawn from the vessel every seventh day, and at the same time freshly prepared medium was added to keep the culture volume constant. Liquid paraffin was layered onto the culture medium to maintain the anaerobic condition. For the extraction of the antiviral substance, cells were harvested by continuous centrifugation, freeze dried and stored at -20°C until used.

Phage stock solution was prepared by the modified method described by Labedan and Legault-Demare (1974). The infection was made in Erlenmeyer flasks (2 litres) containing 200 ml of F medium at 37°C, with a fast agitation on a gyratory shaker. When the bacterial concentration attained 3×10^8 cells/ml, the phage was added at a multiplicity of infection of about 0.1. The infected E. coli was further incubated for 2 h, followed by an addition of chloroform. The bacterial debris was removed by centrifugation (1500xg) and the supernatant was kept at 55°C for 30 min. The phage stock was stored at 4°C. Phage concentration varied from 10^6 to 10^7 plaque forming units (PFU) per ml, however, over this range there was no significant difference in results.

Removal of coliphages from wastewater effluent

Removal of coliphages from wastewater during purification treatment using R. capsulata was measured. The scheme of the purification steps is shown in Fig. 1. The high concentration waste solution (the law feces) and the final effluent were harvested by the grab sample method from a water treatment plant at a commercial pigpen. The samples were brought on ice to the laboratory for the coliphage analyses. Estimation of coliphage was done by the agar layer method in two replicates.

An experimental model of a water purification plant

The wastewater treatment was simulated with five 500 ml tall form beakers and an air pump, with the presence or absence of R. capsulata. Porcine feces were processed every day for over one month to stabilize the microbial population in every beaker. Porcine feces (100 g), harvested at a commercial pigpen, were agitated in water (200 ml) and then kept still for several minutes to settle suspended solids. The supernatant was aerated at 1800 l/l/h(litre air per litre solution per hour) for 24 h in one of the beakers which served as the aeration tank. Next, the solution was divided into two portions; each portion added to a solution in one of two succeeding beakers which served as a culture tank and a control, i.e. with and

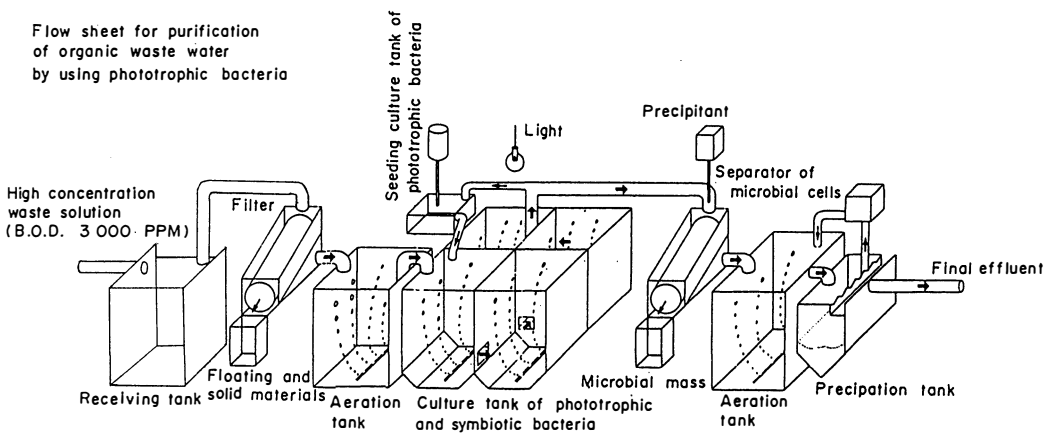

Fig. 1. Flow sheet for purification of organic wastewater by using R. capsulata

without inoculation of cultured R. capsulata every three days. Each of the beakers was placed in a different chamber to avoid contamination from the cultured bacteria. Solutions in the culture tank and the control tank were aerated at 180 l/l/h. When waste solution was moved from the first aeration tank to the culture tanks, 50 ml portions of the existing solutions in the culture tanks were moved to the succeeding beakers. The beakers were aerated at 180 l/l/h, and served as the final aeration and precipitation tanks. The frequency of moving 50 ml portions gave a turnover rate of five days in the culture tanks. At hours 0, 6, 12, and 24 after transfer from the first aeration tank, solutions were collected from the culture tanks for assay of surviving coliphage. The assay was done by the agar layer method in three replicates with peptone-KCl broth which contained 10 g bacto-peptone, 5 g KCl, and 0.075 g $CaCl_2$ per litre(pH 6.8).

Evaluation of antiviral extract from R. capsulata

Chloroform-methanol (2-1) extract from freeze-dried R. capsulata cells (2 g) was saponified for 18 h with 2 M KOH (85% methanol solution) at 65°C. Unsaponifiable substances were removed from the solution with petroleum ether. The remaining aqueous phase, then adjusted to pH 2.0 with 2 N HCl, was extracted with petroleum ether. Petroleum ether was thoroughly evaporated and the remaining fraction was submitted to T5 phage inactivation tests. (Kobayashi et al., 1987b)

T5 phages were kept in contact with the R. capsulata extract (200 μg/ml) for 30 min at 25°C in phosphate buffered saline (PBS) with the presence of 10 mg/ml kaolinite (Kaolin, Nacalai Tesque Co., Japan). PBS contained 4.68 g NaH_2PO_4, 8.66 g Na_2HPO_4, and 8.5 g NaCl per litre (pH 7.0). Phages surviving after the treatment were assayed by the agar layer method in two replicates.

The agar layer method

Titres of T5 phage and coliphages were assayed by the agar layer method. Test solutions (0.5 ml), serially diluted to give 50 to 500 plaque counts per plate, were mixed with enriched culture solutions (0.5 ml) of E. coli strain B in 2 ml of melted soft agar (peptone-KCl broth with 0.5% agar). The mixture was poured onto a previously hardened agar plate (peptone-KCl broth with 1.5% agar). Numbers of turbid plaques obtained on layers of the host bacteria, after incubation for 18 h at 37°C, corresponded to the numbers of the surviving phages.

RESULTS AND DISCUSSION

Coliphage in the wastewater from a commercial pigpen was enumerated to establish the phage removing capacity of the water purification process using R. capsulata. The PFU (plaque forming units / ml sample solution) of raw and treated wastewater were 1.0×10^4 and 2.6×10^2, respectively, suggesting the removal of coliphage during the process.

An experimental model was created to ensure that coliphage removal during wastewater treatment at a pigpen resulted from the presence of R. capsulata biomass. The model plant consisted of bacterial purification steps with and without inoculation of R. capsulata. Since R. capsulata is a facultative anaerobe, a vigorously aerated medium supported good growth of the bacteria. With fresh, growing R. capsulata present, 80.3 % of the coliphages originating from porcine feces were removed within 24 h, thus showing a significant difference with the removal detected without inoculation of the bacteria (Fig. 2). Coliphage population in the tank without inoculation of R. capsulata may have reached a stable state in 6 h, whereas coliphages in the tank with inoculation were continuously removed during the treatment.

Presence of kaolin did not affect T5 phage inactivation with R. capsulata extract (TABLE 1), although it is well known that suspended solids in aquatic systems display a protective effect toward viruses on chlorination (Sproul, 1976; Rao et al., 1984), the most popular disinfection method to date. To wastewater generally containing large amounts of particulate, the efficiency of the extract is suggested, although it does not seem practical to use the extract as a wastewater additive. However, at a wastewater processing plant with an R. capsulata biomass, the bacteria may excrete an antiviral substance into the surrounding wastewater (Kobayashi et al., 1987b). Another superiority of R. capsulata extract over

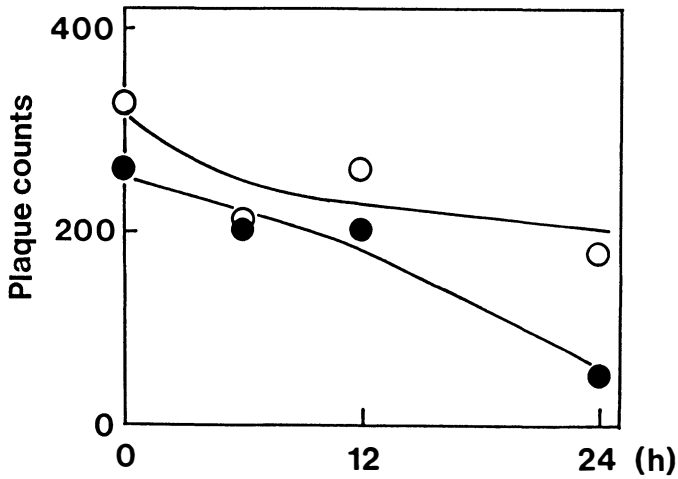

Fig. 2. Inactivation of fecal coliphages during the wastewater treatment with (●) and without (○) R. capsulata biomass

chlorine is its stability to pH changes between 6 to 9 (Fig. 3). Inactivation capacity of chlorine depends on the form of chlorine (Weidenkopt, 1985), which is readily altered by pH levels of the solution. Although the inactivation observed by R. capsulata was less than one order of magnitude, the authors assume this was due to consumption of antiviral substance during the inactivation (Stagg, 1982). The wastewater management plant is usually a huge reactor with continuous flow of the wastewater, which enables constant excretion of antiviral substance from the bacteria. Thus in a wastewater processing plant the inactivation would proceed without decrement of the concentration of the substance and a higher degree of inactivation as described before in the text might have occurred.

A wastewater purifying process using R. capsulata, which is already built and in operation, removed coliphages in the wastewater effluent. This was due to the growing bacteria in the tank. Since the extract from the bacterium inactivates several pathogenic viruses, the purifying process is assumed to remove pathogenic viruses also, when they are contaminated in the effluent. This wastewater purification method seemed more effective than the conventional methods for its ability to remove viruses.

There is a rising concern over the spread of waterborne viruses through food (Tierney et al., 1977; Ward and Irving, 1987) and drinking water. Wastewater processing with a procedure using R. capsulata may help decrease the health risks caused by viruses spread through wastewater. Persistence of viruses in wastewater must be further examined.

TABLE 1 T5 Phage Inactivation with Extract from R. capsulata with the Presence of Kaolinite

	Plaque counts / plate ‹mean›						Inactivation
	Without extract			With extract			
Without kaolinite	112	124	‹118›	62	70	‹66›	44.1%
With kaolinite	107	97	‹102›	49	63	‹56›	45.1%

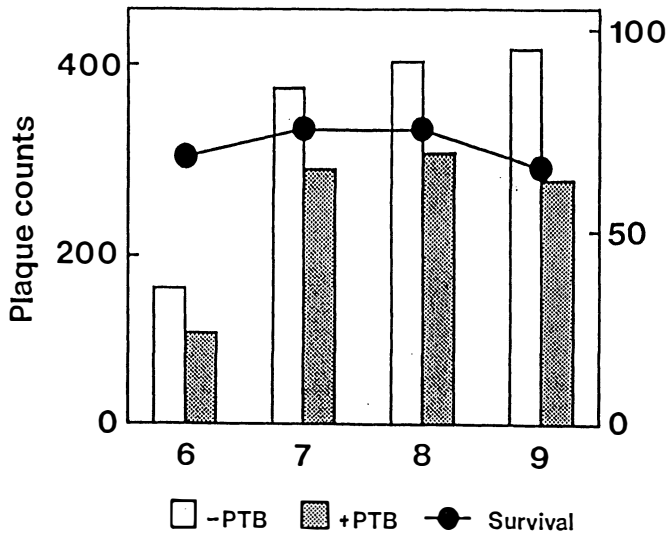

Fig. 3. T5 phage survival at various pH with R. capsulata extract

REFERENCES

Adams, M. (1950). Methods of study of bacterial viruses. Methods Med. Res., 2, 5-11.

Kobayashi, M. and Tchan, Y. T. (1973). Treatment of industrial waste solutions and production of useful by-products using a photosynthetic bacterial methods. Wat. Res., 7, 1219-1224.

Kobayashi, M., Fujii, K., Shimamoto, I., and Maki, T. (1978). Treatment and re-use of industrial waste water by phototrophic bacteria. Prog. Wat. Tech., 11(1), 279-284.

Kobayashi, M. and Hirotani, H. (1987a). Production of antiviral substance by phototrophic bacteria. Soil Microorganisms (in Japanese), 30, 43-48.

Kobayashi, M., Hirotani, H., and Takahashi, E. (1987b). Antiviral substance from phototrophic bacteria. Abstracts of the VII International Congress of Virology, August 9-14, Edmonton, Canada.

Labedan, B. and Legault-Demare, J. (1974). Evidence for heterogeneity in populations of T5 phage. J. Virol., 13, 1093-1100.

Rao , V. C., Seidel, K. M., Metcalf, T. G., and Melnick, J. L. (1984). Appl. Environ. Microbiol., 48, 404-409.

Sproul, O. J. (1976). Removal of viruses by treatment processes. In: Viruses in Water, Berg, G., Bodily, H. L., Lennette, E. H., Melnick, J. L., and Metcalf, T. G. (Ed). American Public Health Association, Washington D. C., pp.167-179.

Stagg, C. H. (1982). Evaluating chemical disinfectants for virucidal activity. In: Methods in Environmental Virology, Gerba, C. P. and Goyal, S. M. (Ed)., Marcel Dekker, New York, pp. 331-348.

Tierney, J. T., Sullivan, R., and Larkin, E. P. (1977). Persistence of poliovirus 1 in soil and on vegetables grown in soil previously flooded with inoculated sewage sludge or effluent. Appl. Environ. Microbiol., 33, 109-113.

Ward, B. K., and Irving, L. G. (1987). Virus survival on vegetables spray-irrigated with wastewater. Wat. Res., 21, 57-63.

Weidenkopt, S. J. (1985). Inactivation of type 1 poliomyelitis virus with chlorine. Virology 5, 56-97.