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COMPARISON OF METHODS FOR THE MEASUREMENT OF GROWTH OF FLOC FORMING *PSEUDOMONAS PUTIDA* CP1 ON MONO-CHLOROPHENOLS

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Abstract

Pseudomonas putida CP1 formed clump of cells when grown on all three mono-chlorophenol isomers**.** EDTA caused no dispersion of flocs. High power (100 W output) sonication caused dispersion of the flocs and also caused cell lysis. Sonication at low power (50 W output) for 30 second caused dispersion without cell lysis. Sodium tripolyphosphate had no significant effect on dispersion of the flocs. Monitoring growth of the organism by a direct microscopic count was found to be more accurate than other methods including optical density measurements, dry weight measurements and the plate count technique.

Introduction

Chlorophenols are a group of xenobiotic compounds of environmental concern. One of the main ways in which they can be removed from the environment is by biodegradation or by biotransformation. However, only a few microorganisms are capable of degrading chlorophenols, so chorophenols persist in the environment causing pollution both in soil and water. *Pseudomonas putida* CP1 is one of the few bacteria capable of degrading all three isomers of mono-chlorophenols (Farrell and Quilty 2002). *P. putida* CP1 forms flocs during growth on mono-chlorophenol containing media, as a result a fairly stable and multicellular association is obtained (Fig. 1). As the flocculation causes problems in sampling, it is difficult to measure the growth of *P.s putida* CP1 by using standard enumeration techniques. Therefore, the bacterium present in the flocs must first be dispersed before the estimation of biomass.

Biomass estimation is important in microbial and other bioprocesses. An accurate method for real-time biomass estimation during a bioprocess is an important goal to be achieved. Despite the many promising classical methods available, evaluation of microbial growth in bioprocesses may sometimes become laborious, impracticable and give erroneous values (Singh *et al.* 1994). Classical methods for biomass determination may be based on cell number or cell mass. Total count usually does not differentiate between active and dead cell populations whereas a few methods may provide the counts of viable or active cells. However, viable counts do not distinguish between cells and clumps of cells. Viable counts usually underestimate the microbial community when compared to direct count methods (White 1988, Herbert 1990).

To enumerate the floc forming bacterium *P. putida* CP1, an attempt was made to deflocculate flocs by using ultrasonic sound at different intensities as well as using deflocculating agent EDTA. The effect of sodium tripolyphosphate on flocs dispersion was also investigated. Attempt was also made to measure the growth of the organism on mono-chlorophenols by using optical density measurements, dry weight measurements, plate count method and direct microscopic count technique.

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Materials and Methods

The isolate *Pseudomonas putida* CP1 was obtained from Dr. Favio Fava, University of Bologna, Italy. *P. putida* CP1 was grown overnight in Nutrient broth, centrifuged at 5000 rpm for 10 min and washed with 0.01 M sodium phosphate buffer. Five ml was used to inoculate 95 ml sterile minimal medium (Goulding *et al.* 1988) containing 200 ppm mono-chlorophenol. After inoculation flasks were incubated in an orbital shaker at 150 rpm at 30^0C. The effect of EDTA on deflocculation was measured by a modified method of Silverstein *et al.* (1994).

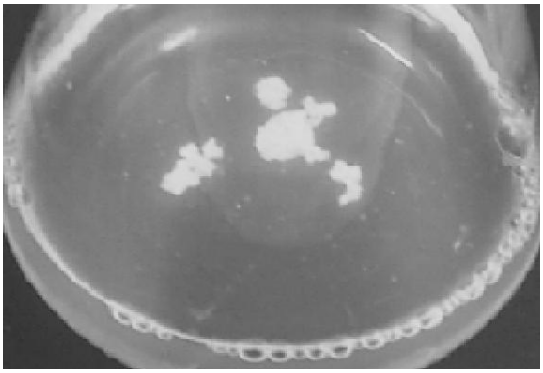

Fig. 1. Formation of flocs by *Pseudomonas putida* CP1 grown on mono-chlorophenol.

Effect of low and high power output sonication for floc dispersion: A modified method of Jorand *et al.* (1995) was used. Flocculated samples were taken from three different flasks and the samples were diluted tenfold. This suspension was taken in a beaker and dispersed with an ultrasound generator (Labsonic 2000 U, Standard 19 mm probe) by using both high (100 W output) and low power (50 W output) for 10 sec, 30 sec, 1 min and 3 min and the effect was observed.

Effect of surfactant (Sodium tripolyphosphate) on deflocculation: Flocculated samples were diluted tenfold with 5 mg/l of sodium tripolyphosphate as well as with distilled water. Undiluted samples were used as control. The samples were sonicated at 50 W for 30 sec and the deflocculation was observed.

Determination of cell lysis for sonicated samples: The method of Biggs and Lant (2000) was used to determine cell lysis where, activity of the intracellular enzyme glucose-6-phosphate dehydrogenase (G6PDH) was measured as an indication of cell lysis.

Measurement of growth of P. putida CP1 on mono-chlorophenols: The flocs were disrupted by using low power (50 W output) sonication for 30 sec prior to measurement of cell numbers. Growth of *Pseudomonas putida* CP1 was monitored by using a number of methods including optical density measurement at 660 nm, dry weight measurement, measurement of bacterial number by the pour plate method and the direct epifluorescence filtration technique.

The determination of P. putida CP1 cell number using epifluorescence microscopy: The samples were collected and immediately diluted tenfold with quarter strength Ringer solution and the viable and nonviable bacterial counts were made according to the method by Bitton *et al.* (1993). Live cells fluorescence orange-red whereas dead cells fluorescence green. The number of

bacteria per ml of sample was calculated using the formula, $T = N \times A/a \div V$ (Boulos *et al.* 1999) where, T = number of bacteria/ml, N = average number of bacteria/field, A = area of the filter (mm^2), a = area of the microscopic field (mm^2), V = volume of the sample filtered (ml).

Pictures for documenting cell shape and size were taken by using a JVC KY-F55B colour video camera (Vistor Company of Japan Ltd., Japan) attached to a Zeiss Axioplan Epifluorescence Microscope equipped with a Zeiss filter 09.

Assay of mono-chlorophenols: Mono-chlorophenols concentrations were determined by using the 4-aminoantipyrene colorimetric method based on the procedure detailed in Standard Methods for the Examination of Water and Wastewater (1998).

Results and Discussion

Effect of EDTA on the deflocculation of P. putida CP1 flocs: Due to the flocculation on mono-chlorophenols, it was difficult to measure growth of *P. putida* CP1 by using conventional procedures, such as optical density measurements. Flocculation caused problems specially in sampling. Therefore, an attempt was made to deflocculate the flocs by using a deflocculating agent ethylene-diamine-tetra-acetic acid (EDTA). It was found that up to 2.5 mM of EDTA there was no deflocculation of flocs, 5 to 20 mM EDTA caused partial deflocculation of flocs. Although EDTA treatment caused partial dispersion of *P. putida* CP1 flocs, it also caused lysis of the cell at a concentration greater than 10 mM. Therefore, the EDTA treatment was not useful for the dispersion of *P. putida* CP1 flocs. EDTA breaks up flocs by tying up multivalent inorganic ions, e.g., calcium, which is responsible for floc aggregation. Silverstein *et al.* (1994) reported that 1% (w/v) EDTA solution was effective in breaking up sludge flocs, but their treatment also resulted in cell lysis.

Effect of low and high power output sonication for floc dispersion: Sonication was found to be the best method for the dispersion of *P. putida* CP1 flocs. Release of bacteria from flocs depends on both the intensity and duration of sonication. High power (100 W) sonication caused dispersion of cells and cell lysis, but sonication at a low power (50 W) for 30 seconds was found to be effective for the dispersions of cells and caused no cell lysis. Therefore, sonication at low power (50 W) for 30 seconds was selected. Several other investigators used sonication as a method for the dispersion of flocs of activated sludge by using comparable intensities and time (King and Forster 1990, Jorand *et al.* 1994, Jorand *et al.* 1995, Snidaro *et al.* 1997). Jorand *et al.* (1994) reported that the sonication of activated sludge at 37 W for 60 seconds was the best method for the dispersion of flocs with minimal cell lysis.

Effect of surfactant (Sodium tripolyphosphate) on deflocculation: An attempt was made to observe the effect of the surfactant sodium tripolyphosphate on deflocculation. There was no significant variation in viable bacterial count among the diluted (either with sodium tripoly-phosphate or with distilled water) and undiluted samples. So sodium tripolyphosphate has no significant effect in the deflocculation of flocs of *P. putida* CP1. There are reports of the use of sodium tripolyphosphate to enhance dispersion of flocs of phenol degrading bacteria (Fujita *et al.* 1994, Soda *et al.* 1998). Pike *et al.* (1972) found that the addition of 5 mg/l sodium tripolyphosphate before homogenization was suitable for the disruption of flocs and the enumeration of aerobic bacteria in activated sludge. However, preliminary studies in this work showed that the surfactant sodium tripolyphosphate had no effect on the dispersion of the *P. putida* CP1 flocs.

Determination of cell lysis in sonicated samples: Activity of intracellular enzyme glucose-6 phosphate dehydrogenase (G6PDH) was used to identify the cell lysis. If the enzyme is detected in the supernatant of culture fluid, cell lysis assumed to have occurred. It was observed that there was no significant release of G6PDH as a result of cell lysis by sonication up to 1 min. Little or no cell lysis occurred by sonication at 50 W up to 60 sec. The physical method of sonication was selected because sample contamination could be avoided and so the subsequent study of various parameters would not be hampered. Biggs and Lant (2000) and Frølund *et al.* (1996) also used a similar technique for the identification of cell lysis in activated sludge.

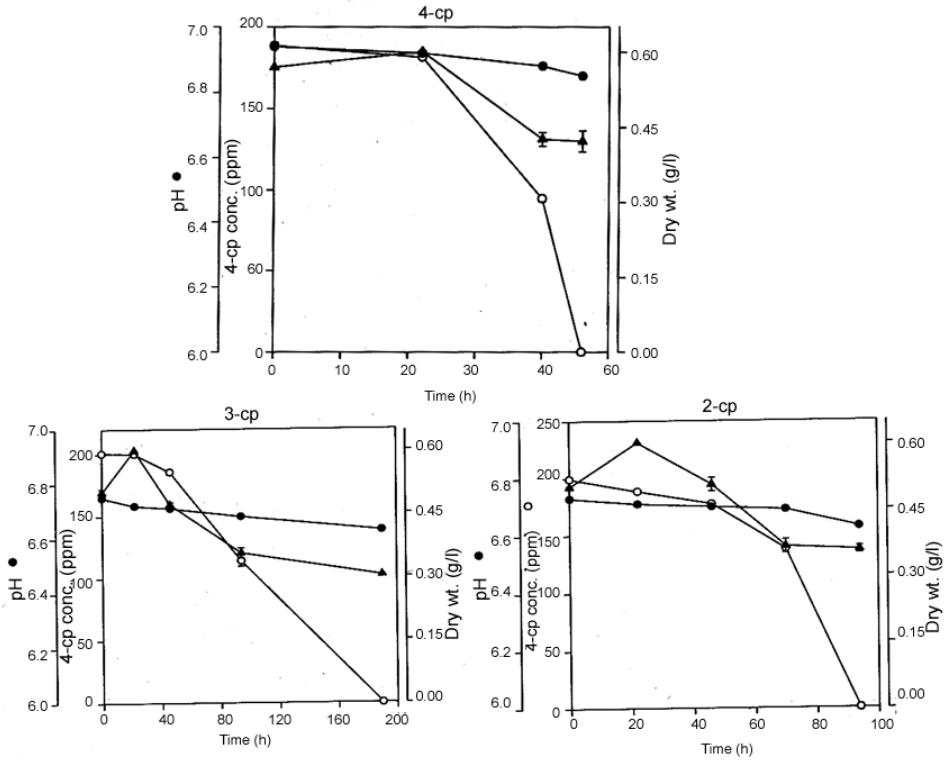

Fig. 2. Changes in mono-chlorophenol concentration, dry weight and pH during growth of *P. putida* CP1 on 200 ppm mono-chlorophenols. Symbols: o = mono-chlorophenol concentration; ● = pH and ▲ = dry weight.

Growth of P. putida CP1 on 200 ppm mono-chlorophenols: Fig. 2 shows the utilization of mono-chlorophenols by *P. putida* CP1 and changes in dry weight and pH during growth of on 200 ppm mono-chlorophenols. The complete removal of 4-, 3- and 2-chlorophenol was found in 46, 190 and 96 h, respectively. The organism flocculated when grown on mono-chlorophenols. There was negligible change in pH. When growth of the organism was monitored using dry weight measurements, the biomass was seen to decrease in value with time as substrate was removed from the medium. The dry weight initially increased slightly but dropped steadily after 22 h. There was a drop of 0.12, 0.21 and 0.16 g/l of biomass with the growth and complete removal of 4-, 3- and 2-chlorophenol, respectively. Thus, the greatest drop in dry weight was found with 3-chlorophenol followed by 2-chlorophenol, followed by 4-chlorophenol. A change in

bacterial cell number during growth on 200 ppm mono-chlorophenols containing minimal medium is shown in Fig. 3. In the case of the standard plate count method the numbers of cells initially decreased. The numbers of cells recovered and increased with time as the mono-chlorophenol was removed from the medium. The numbers of acridine orange stained viable cells observed using the direct epifluorescence filtration technique was always greater than the numbers counted using the standard plate count method. No non-viable cells were noted until the end of a run when the particular mono-chlorophenol was removed from the medium.

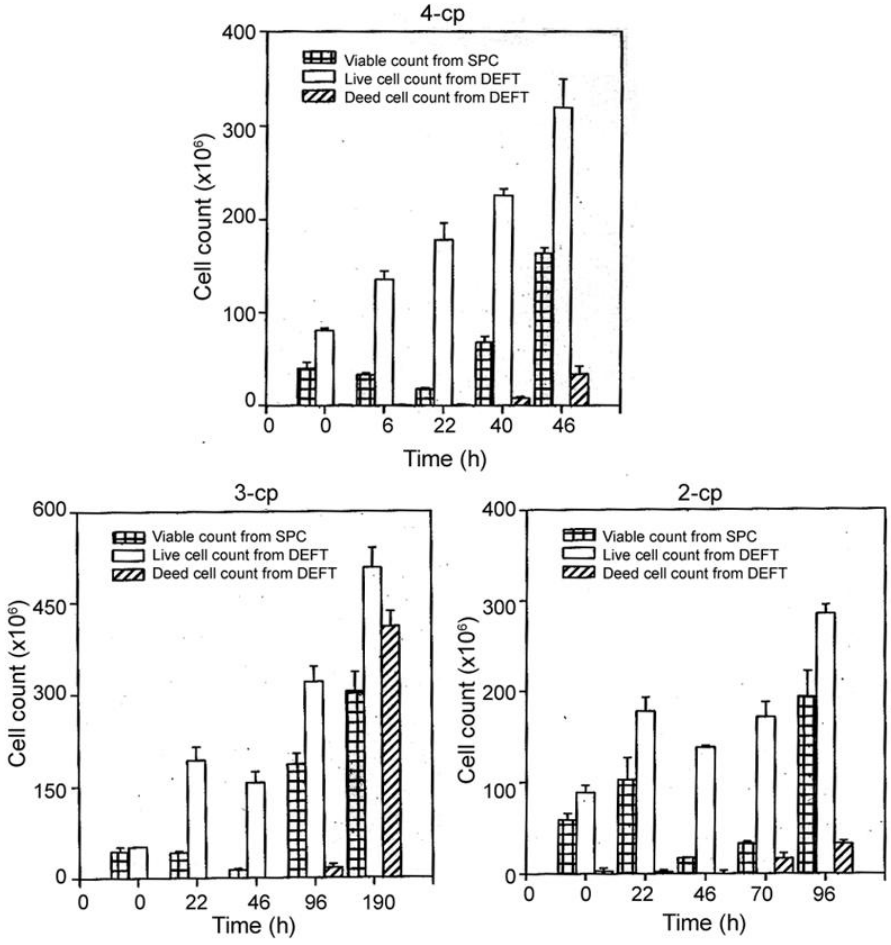

Fig. 3. Measurement of growth of *P. putida* CP1 on 200 ppm mono-chlorophenols by the standard plate count and the direct epifluorescence filtration.

When *P. putida* CP1 was grown on mono-chlorophenols variations in cell numbers were found between the standard plate count (SPC) and the direct epifluorescence filtration technique (DEFT). This may be due to the presence of viable but non-culturable cells of *P. putida* CP1. Direct cell counts of bacteria in water and wastewater usually exceed counts obtained from heterotrophic plate counts and the most probable number method because unlike those procedures, direct counts preclude errors caused by viability-related phenomena such as

selectivity of growth media, cell clumping, and slow growth rates (Standard Methods for the Examination of Water and Wastewater 1998).

Conventional culture techniques have been shown to have many shortcomings. This study highlights the value of using the direct epifluorescent filtration technique for real time measurement of biomass in environmental samples. The direct epifluorescence filtration technique is a rapid and sensitive enumeration technique, which allows microscopic counting of bacteria retained on a filter (Hobbie *et al.* 1977, Bittton *et al.* 1993, Standard Methods for the Examination of Water and Wastewater 1998). Monitoring growth is frequently done by measuring dry weight. In this study it was noted that an increase in cell numbers for the organism grown on mono-chlorophenols was accompanied by a decrease in the dry weight. Microscopic examination showed that the shape of the bacterial cell changed from rod shape to coccus shape when the organism was grown on mono-chlorophenols. In these instances, not only did the cell change shape but also the volume of the cell decreased. This explained the decrease in dry weight while there was an increase in cell number and again highlighted the need for real time measurement of cell numbers.

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