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Measurement of the growth of a floc forming bacterium *Pseudomonas putida* CP1

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Abstract *Pseudomonas putida* CP1 formed clumps of cells when grown on mono-chlorophenols but not on phenol or glucose. An increase in cell numbers for the organism grown on mono-chlorophenols was accompanied by a decrease in the dry weight. The change in shape of the bacterium from rod shape to coccus shape coupled with a reduction in cell size when the organism was grown under nutritional stress was found. This result together with cell aggregation affected the measurement of growth parameters in the system by conventional methods (optical density measurements, dry weight measurements and the plate count technique). Monitoring growth of *Pseudomonas putida* CP1 by a direct microscopic count technique was found to be more representative than conventional methods including optical density measurements, dry weight measurements and the plate count technique when grown on phenolics.

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Introduction

Biomass estimation is important in microbial and other bioprocesses. Its determination leads to an understanding of the efficiency of a biological system. Although biomass concentration is a simple measure, it is a key variable in measuring rates of growth and product synthesis, yield coefficients, and also for the calculation of specific rates and mass balance in any bioprocess. The importance of quantifying the organisms responsible for degradation of toxic compound for elucidation of microbial degradation kinetics, developing biological treatment process model or defining microbial population dynamics were pointed out (Chudoba et al. 1989; Schmidt and Gier 1989; Wanner and Elgi 1990; Silverstein et al. 1994). Thus, 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. Methods dependent on cell number are observational, based on physical and microbial

activity. These methods include total and viable counting of cells. 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).

Pseudomonas putida CP1 formed clumps of cells when grown under certain environmental conditions (Farrell and Quilty 2002). This caused difficulties for monitoring growth. Clump formation caused problems specially in sampling. The aims of this project were to compare various methods for assessing the growth and growth parameters of the floc forming bacterium *P. putida* CP1.

Materials and methods

Source of *P. putida* CP1

The isolate *P. putida* CP1 was obtained from Dr. Favio Fava, University of Bologna, Italy. It was originally isolated from soil in the United States and the identification described elsewhere (Farrell 2000; Farrell and Quilty 2002).

Meida

Pseudomonas minimal medium

The ingredients of the minimal medium (Goulding et al. 1988) were combined in distilled water and the pH was adjusted to 7.0 with 2 M NaOH. The trace salts solution was prepared separately in distilled water and was stored in a dark bottle for 6–8 weeks. The ingredients of the per litre minimal medium are as follows: (K_2HPO_4, 4.36 g; NaH_2PO_4, 3.45 g; NH_4Cl, 1.0 g; $MgSO_4 \cdot 6H_2O$, 0.912 g; pH, 7.0). Trace salts solution was added at a concentration of 1 ml per litre. The composition of the per 100 ml trace salts solution was as follows: ($CaCl_2 \cdot 2H_2O$, 4.77 g; $FeSO_4 \cdot 7H_2O$, 0.37 g; $CoCl_2 \cdot 6H_2O$, 0.37 g; $MnCl_2 \cdot 4H_2O$, 0.10 g; $Na_2MoO_4 \cdot 2H_2O$, 0.02 g). The mono-chlorophenols/phenol were added to the minimal medium after sterilization.

Chlorophenol/phenol agar

Pseudomonas minimal medium was prepared as described above. Bacteriological agar (Oxoid) at a concentration of 1% (w/v), was added to the minimal medium. Immediately prior to pouring, chlorophenol/phenol was added to the media to give the appropriate concentrations. Chlorophenol/phenol broth was used for biodegradation studies, the composition of which was exactly similar to the chlorophenol/phenol agar except that no agar was added to it.

Maintenance of *P. putida* CP1

The bacterium was maintained on chlorophenol agar. The organism was kept at 4°C up to 1 month and then sub-cultured. For long term storage the organism was lyophilized.

Culture conditions

Pseudomonas putida CP1 was grown overnight in nutrient broth, centrifuged at 5,000 rpm for 10 min and washed twice with 0.01 M sodium phosphate buffer (pH 7.0) and then resuspended to give an optical density of 0.7 at 660 nm. Five millilitre of culture suspension was used to inoculate 95 ml sterile minimal medium (Goulding et al. 1988) containing mono-chlorophenols (200 ppm) and phenol (500 ppm) in 250 ml conical flasks. Thus, the flasks were inoculated with a 5% (vol/vol) inoculum, which corresponded to $(3.5 \pm 0.5) \times 10^7$ cells/ml. The concentrated stock solution of the glucose was separately sterilized prior to their addition to the sterilized minimal medium to give the appropriate final concentration, where the glucose effects were being studied. After inoculation, flasks were incubated in an orbital shaker at 150 rpm at 30°C. Uninoculated control flasks were run in parallel. For non-flocculated growth, samples were aseptically removed at regular intervals and analyzed for growth and pH. For flocculated growth, 100 ml of minimal medium containing flocculated *P. putida* CP1 cells was taken in a glass beaker and dispersed with an ultra sound probe (Labsonic 2000 U, Standard 19 mm probe) by using low power (50 W output) for 30 s before the measurement of cell number and pH. Samples were then centrifuged at 5,000 rpm for 10 min, the

supernatants were then analyzed for phenol/chlorophenol removal and for glucose where appropriate.

Measurement of growth of *P. putida* CP1

Growth of *P. 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 microscopic method for the determination of both viable and non-viable cells. *Pseudomonas putida* CP1 flocs were disrupted by sonication prior to measurement of cell numbers and deflocculation was observed microscopically. The method of Biggs and Lant (2000) was used to determine cell lysis after sonication, where the activity of the intracellular enzyme glucose-6 phosphate dehydrogenase (G6PDH) was measured as an indication of cell lysis.

Measurement of bacterial numbers by the pour plate method

Samples were serially diluted and poured in triplicate using plate count agar. Plates were incubated in 30°C for 2 days and counted. The bacterial count was expressed as colony forming units per ml (cfu/ml).

Dry weight measurement

For non-flocculated growth, a specific volume of suspended culture was filtered through two tarred filters (Whatman GF/C and Gelman 0.2 μm, 47 mm membrane filters). For flocculated growth, entire content in the flasks (100 ml of minimal medium containing flocculated *P. putida* CP1 cells) were filtered through two tarred filters. Filters were dried at 85°C for 2 h and then reweighed.

Turbidity measurement

Growth was monitored turbidimetrically by noting the optical density (OD) at 660 nm using a Unicam UV/VIS spectrophotometer.

The determination of P. putida *CP1 cell number using epifluorescence microscopy*

The samples were collected and immediately diluted 10-fold with quarter strength Ringer Solution and the viable and non-viable bacterial count was determined according to the method described by Bitton et al. (1993). One millilitre of the diluted suspension was mixed with 1 ml of 0.1% (w/v) acridine orange solution. After a 2 min contact time, the bacteria were recovered by vacuum filtration through 0.2 μm black polycarbonate membrane filters (Millipore GTBP 04700). At least 10 randomly selected fields containing 10–50 cells were examined on the filter using the 100× oil immersion lens. Live cells fluorescence orange-red whereas dead cells fluorescence green. The number of bacteria per ml of sample was calculated using the formula (Boulos et al. 1999). 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 at 1,000× magnification.

Assay of mono-chlorophenols and phenol concentration

Mono-chlorophenols and phenol 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).

Reducing sugar determination

The glucose concentrations were determined by the dinitrosalicylate (DNS) colorimetric method (Miller 1959).

Calculation of growth yield

Yield was calculated using either the dry weight (mg) of cells or the number of cells obtained by using the standard plate count method and the direct epifluorescence filtration technique. When the change in dry weight (mg) of the organism was used to calculate yield, the result was expressed as mg of dry cell weight produced per mg of substrate utilized. When cell number was used to calculate growth yield, the yield of the organism was expressed in terms of number of cells produced per mg of substrate utilized.

Calculation of specific growth rate

Specific growth rates were determined by plotting the log of cell number against time during exponential growth. The regression coefficient and slopes were calculated using a Sigma Plot, Version 1.02 Computer Package (Jandal Scientific Corporation). The resulting slope being equal to the specific growth rate, μ.

Data analysis

The results presented were the mean of duplicate treatments and all experiments were repeated to confirm the data obtained. Standard errors were determined using a Sigma Plot. In all cases, the standard errors between runs were found to be less than 5%.

Results

Measurement of growth of *P. putida* CP1 on 200 ppm mono-chlorophenols and on 500 ppm phenol

Figure 1 shows the utilization of mono-chlorophenols and phenol by *P. putida* CP1 and changes in dry weight during growth on 200 ppm mono-chlorophenols and 500 ppm phenol. The removal of mono-chlorophenols was preceded by a lag period, which varied for different isomers. Following lag the organism was able to degrade completely 200 ppm 4-chlorophenol, 3-chlorophenol and 2-chlorophenol 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 but dropped steadily after 22 h. There was a drop of 0.136, 0.206 and 0.145 g/l of biomass with the growth and complete removal of 4-, 3- and 2-chlorophenol, respectively (Table 1). Thus, the greatest drop in dry weight was found with 3-chlorophenol followed by 2-chlorophenol, followed by 4-chlorophenol. Unlike with dry weight, a positive response was obtained for cell number when the organism was grown on the mono-chlorophenols. The greatest increase in cell number was found with 3-chlorophenol followed by 2-chlorophenol, followed by 4-chlorophenol.

When 500 ppm phenol was used as the sole carbon source for *P. putida* CP1, no flocculation of the cells was observed. Figure 1 shows the changes in phenol concentration and dry weight during growth of *P. putida* CP1 on 500 ppm phenol. There was negligible change in the pH of the medium. The increase in dry weight as well as cell number was observed during the growth of *P. putida* CP1 on 500 ppm phenol.

Measurement of growth of *P. putida* CP1 on 0.5% (w/v) glucose and on 500 ppm phenol plus 0.5% (w/v) glucose

No clumping of the organism was observed when the organism was grown on 0.5% (w/v) glucose. The organism utilised the glucose readily and there was a linear removal of substrate. The glucose was completely removed in 22 h. There was 0.6 unit drop in pH on 0.5% (w/v) glucose containing media. Increase in dry weight was observed during growth of *P. puitda* CP1 on glucose (Table 1).

The two substrates were removed concurrently from the medium, when the organism was grown on 500 ppm phenol plus 0.5% (w/v) glucose. The organism grew in response to substrate removal and this was recorded as an increase in dry weight (Table 1). There was 0.50 unit change in pH observed in the presence of both substrates. The complete removal of both glucose and phenol was found in 46 h.

The determination of growth parameters of *P. putida* CP1 using various approaches

Pseudomonas putida CP1 was grown on various substrates and the growth response was measured in terms of dry weight and cell number and used to calculate the growth rate, μ (Table 1) and the growth yield, Y (Table 2). In the case of dry weight, while there was an increase in dry weight when the organism was grown on glucose (0.5%, w/v), 500 ppm phenol and glucose plus phenol, while growth on the mono-chlorophenols (200 ppm) resulted in a decrease in dry weight of the organism. The greatest increase in dry weight was obtained

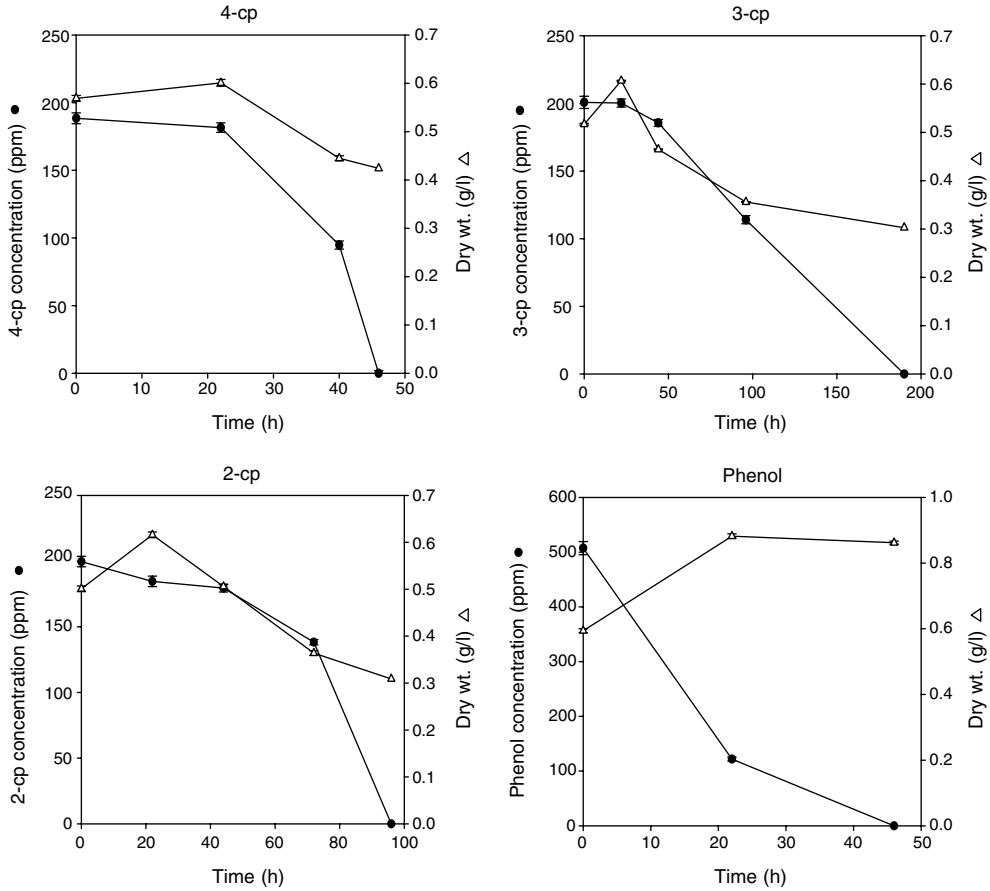

Fig. 1 Changes in mono-chlorophenols or phenol concentrations and dry weight during growth of *P. putida* CP1 on 200 ppm mono-chlorophenols or 500 ppm phenol at 30°C

Table 1 Growth characteristics of *P. putida* CP1 on 200 ppm mono-chlorophenols, 500 ppm phenol, 0.5% (w/v) glucose and 0.5% (w/v) glucose plus 500 ppm phenol

Substrate	Time (h)	Δ Cell dry weight (g/l)	Δ Cell no. SPC × 10^8 (cfu/ml)	Δ Cell no. (DEFT)		Growth rate, μ (h^{-1})		
				Viable(×10^8)	Total(×10^8)	SPC	DEFT (viable)	DEFT (total)
200 ppm								
4-chlorophenol	46	−0.136 ± 0.011	1.25 ± 0.06	2.39 ± 0.12	2.59 ± 0.165	0.013	0.017	0.018
3-chlorophenol	190	−0.206 ± 0.001	2.59 ± 0.02	4.54 ± 0.02	8.66 ± 0.245	0.0057	0.0061	0.0065
2-chlorophenol	96	−0.145 ± 0.009	1.35 ± 0.20	1.96 ± 0.10	2.29 ± 0.125	0.006	0.0061	0.0066
500 ppm phenol	22	+0.269 ± 0.014	6.88 ± 0.12	7.22 ± 0.30	10.20 ± 0.03	0.059	0.064	0.068
0.5% (w/v) glucose	22	+1.36 ± 0.029	16.30 ± 0.18	18.36 ± 0.10	18.64 ± 0.10	0.082	0.086	0.087
0.5% (w/v) glucose+ 500 ppm phenol	46	+0.788 ± 0.023	7.84 ± 0.11	10.8 ± 0.20	11.4 ± 0.025	0.067	0.069	0.071

±, Standard error; SPC, standard plate count; DEFT, direct epifluorescence filtration technique

when the organism was grown on glucose, followed by phenol and glucose combined and then phenol alone. This trend was also obtained when growth was measured using cell number. The greatest increase in cell number was obtained when the organism was grown on glucose. The difference in the number of

Table 2 Growth yield of *P. putida* CP1 when grown on 200 ppm mono-chlorophenols, 500 ppm phenol, 0.5% (w/v) glucose and 0.5% (w/v) glucose plus 500 ppm phenol

Substrate	mg/mg	Growth yield, Y		
		$\times 10^8$ cell/mg		
		SPC	DEFT (viable)	DEFT (total)
200 ppm				
4-chlorophenol	−0.721 ± 0.003	8.20 ± 0.75	14.2 ± 0.06	15.6 ± 0.48
3-chlorophenol	−1.036 ± 0.002	14.6 ± 0.04	23.4 ± 0.66	44.5 ± 2.80
2-chlorophenol	−0.726 ± 0.005	8.10 ± 0.05	9.6 ± 0.07	12.5 ± 0.70
500 ppm phenol	+0.681 ± 0.001	17.81 ± 0.76	20.62 ± 0.82	26.72 ± 1.76
0.5% (w/v) glucose	+0.391 ± 0.004	5.00 ± 0.04	6.13 ± 0.05	6.78 ± 0.06
0.5% (w/v) glucose+500 ppm phenol	+0.176 ± 0.000	2.10 ± 0.02	2.45 ± 0.04	2.5 ± 0.04

±, Standard error; SPC, standard plate count; DEFT, direct epifluorescence filtration technique

cells when the organism was grown on phenol and phenol plus glucose was more accurately determined when the direct cell count method was used. The growth rate (μ) of the organism was calculated in three ways using the number of cells as determined by the standard plate count method and the direct epifluorescence filtration technique (Table 1). The highest value was obtained when the organism was grown on glucose, then glucose with phenol, followed by phenol and the chlorophenols. In the case of chlorophenols, the highest growth rate was obtained with 4-chlorophenol. Similar values were obtained with 3-chlorophenol and 2-chlorophenol.

The method of measuring cell growth influenced the calculation of yield (Table 2). When the change in dry weight of the organism was used, the yield was greatest when the organism was grown on phenol followed by glucose and then phenol with glucose. Negative values were obtained for the organism when grown on the mono-chlorophenols. When cell number was used to calculate growth yield the value varied depending on the method used for the measurement of cell numbers. The greatest growth yield in terms of cell number was found with 3-chlorophenol, when the direct epifluorescence filtration technique was used to measure cell number. But the greatest growth yield in terms of cell number was found with phenol, when the standard plate count was used to measure cell number.

Discussion

Pseudomonas putida CP1 formed flocs during growth on mono-chlorophenols and it was difficult to measure growth by using conventional growth measurement procedures, such as optical density measurements. The physical method of sonication was selected because sample contamination could be avoided and subsequent study of various parameters would not be hampered. Sonication at a low power (50 W) for 30 s was found to be effective for the dispersions of cells of *P. putida* CP1 and caused little or no cell lysis. 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, 1995; Snidaro et al. 1997). Jorand et al. (1994) reported that the sonication of activated sludge at 37 W for 60 s was the best method for the dispersion of flocs with minimal cell lysis.

When *P. putida* CP1 was grown on a variety of substrates, variations in cell numbers were found between the standard plate count (SPC) method 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).

This study highlights the value of using the direct epifluorescent filtration technique for real time measurement of biomass in environmental samples. 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. Image analysis showed that the shape of the bacterial cell changed from rod shape to coccus shape when the organism was grown under certain environmental stress. In certain 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. Givskov et al. (1994) also reported changes in cell shape and size leading to an increase in cell number but not an increase in cell mass with *P. putida* KT2442. A decrease in cell size accompanied by an increase in population during starvation of marine bacteria was reported by Amy and Morita (1983) and Novitsky and Morita (1976). Size reduction was also reported for starved cells of *P. aeruginosa* and *P. fluorescens* by Mueller (1996) and Makarov et al. (1998). Shrinkage in size due to starvation was also reported for *Pseudomonas* sp. strain A by Sanin et al. (2003). Electron micro-photographic studies of their work showed that *Pseudomonas* sp. strain A tended to increase it's surface area to volume ratio as a starvation response and increased the ability to transport nutrients into the cell with minimum energy consumption. The morphologic and structural variations suffered by cells of a population of *P. aeruginosa* ATCC 27853 under stress conditions were investigated by using scanning near-field optical microscopy (Cefalì et al. 2002). They described that in the structural and morphological modification of rod shaped bacteria to coccoid shape cellular matter seemed to rearrange itself to attain a coccoid stress resistant form, responsible for the residual viability of the population. Several others investigators reported the changes in cell shape of *Escherichia coli* and *Vibrio* from cylindrical to small spheric during the transition phase between growth and non-growth phases to adjust their metabolic rate to a lower level (Kjelleberg et al. 1987; Nyström et al. 1991; Siegele and Kolter 1992). Thus it is suggested that the findings from the present study show that the round morphology can be assumed by the rod-shaped bacterium *P. putida* CP1 as a consequence of alterations in the shape determining mechanism due to the nutritional stress.

Brettar et al. (1994) studied the survival and fate of *P. putida* DSM 3931 in lake water. They released TOL-plasmid bearing *P. putida* as a representative of xenobiotic-degrading bacterium and the study was carried out in mesocosms with unamened lake water and in lake water with added culture medium to compare the survival of strain due to the influence of different organic load. They described that experimental tools on a mesoscale are crucial in order to understand the complex processes micro-organisms are subjected to after release into the natural environment, and that the single cell detection, such as immunofluorescence, is essential to understand the mechanisms of survival and elimination.

Pirt (1975) and Isken et al. (1999) reported yield values for bacteria grown on glucose in terms of g/g comparable to those values obtained in this study. The growth yield data for the growth of CP1 on phenol 0.68 mg/mg was also comparable to the data from previous investigators for other *P. putida* strains which ranged from 0.52 to 0.80 g/g. (Hill and Robinson 1975; Yang and Humphrey 1975; Kotturi et al. 1991; Dikshitulu et al. 1993; Şeker et al. 1997; Reardon et al. 2000). Growth yield of the organism was also expressed in terms of numbers of cells produced per mg of substrate utilized. This approach has also been reported in the literature (Shreve and Vogel 1993; Frías et al. 1994). Shreve and Vogel (1993), reported the yield coefficient of 1.44×10^{10} cells/mmol for toluene grown cells of *Pseudomonas* sp. strain K3-2. A value comparable to that was obtained when *P. putida* CP1 was grown on phenol and 2-chlorophenol. In this study, it was determined that growth on toxic substrates was more accurately measured using a direct cell count method such as the direct epifluorescence filtration technique (DEFT). This needs to be considered when evaluating yield and other growth parameters when cultivating bacteria on toxic substrates.

Conclusions

An increase in cell numbers for the organism grown on mono-chlorophenols was accompanied by a decrease in the dry weight. A change in cell shape from rod to coccus as well as reduction in cell size was also noted when the organism was exposed to

nutritional stress. Monitoring growth of the bacterium under conditions of environmental stress was best achieved using real time measurement, the direct epifluorescence filtration technique was found to be suitable.

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