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# Growth and enumeration of the meat spoilage bacterium *Brochothrix thermosphacta*

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**Abstract**

*Brochothrix thermosphacta* is a common meat spoilage bacterium. The morphology of this bacterium changes from coccobacilli and short rods to chains during growth, which may give a false estimation in numbers using some enumeration techniques. Methods for the quantification of this bacterium have been compared. Turbidimetric readings showed good agreement with cell dry weight indicating that the former provides a good measure of the change in cell mass during growth. The turbidimetric method also correlated well with bacterial numbers determined by plate counts, flow cytometry and manual counts (by microscope) over a limited range of $10^7 - 10^9$ cells/ml. Flow cytometry and manual counts gave a linear relationship over a wider range of $10^5$–$10^9$ cells/ml. The sensitivity of analysis, growth rates and lag time attained using these methods were also compared. As a consequence of changes in bacterial cell size during growth, turbidimetry over-estimated the growth rate. The plate count method proved unable to detect the difference between bacteria existing as chains or single cells. The sensitivity of analysis and the calculated growth related parameters were similar for flow cytometry and manual counts. This suggests that flow cytometry is capable of counting individual cells in a chain. Further investigation showed that passage of *B. thermosphacta* cells through the flow cytometer resulted in the breakage of chains into single cells. The reliability, low error and rapidity of this technique make it attractive for bacterial enumeration, something which has been demonstrated using *B. thermosphacta*, a bacterium which exhibits complex morphologies.


*Keywords:* *Brochothrix thermosphacta*; Bacterial morphology; Flow cytometry; Turbidimetry

## 1. Introduction

For industrial food production, the prevention of contamination, destruction of microbes or adjustment of the condition of foods to prevent microbial growth during storage are usually necessary. However, under many circumstances, aseptic handling conditions are impossible. For food microbiologists, the accurate quantification of micro-organisms is necessary for two different reasons. Firstly, for quality control

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purposes to monitor changes in the population of micro-organisms during food processing (Mol et al., 1971; Laplace-Builhé et al., 1993). Secondly, growth data derived from laboratory experiments using different environmental conditions (such as pH and temperature) can be used to develop mathematical models to predict bacterial growth rates in real foods (Sutherland and Bayliss, 1994; Sutherland et al., 1994).

Several different methods can be used for microbial enumeration. Turbidimetry is the most convenient and inexpensive method and can be related to dry weight by a linear relationship (Dalgaard et al., 1994). However, it is affected by cell size and shape and is not practical for cell clumps or for suspensions containing fewer than $10^7$ cells/ml (Pery and Staley, 1997). Another commonly used method is plate counts which can measure the reproductive viability of cells. A disadvantage of this technique includes the possibility that a colony may come from a clump of cells, resulting in an under estimation of cell numbers (McMeekin et al., 1993). Moreover, the plates need a long period of incubation, around 24–72 h, which is not suitable for short shelf-life products (Laplace-Builhé et al., 1993).

Many rapid methods have been developed to solve these problems and flow cytometry (FC) has attracted much interest. It is an automated technique in which cell suspensions are passed through a laser beam and quantified in flow using electrical and optical detectors (Shapiro, 1988). Furthermore, cellular features can be labelled using fluorochrome markers, thereby enabling one to obtain further information about the micro-organisms under investigation. Hence, flow cytometry has been used widely for studying cell size and structure, the quantification of cellular components, cell physiological activity estimation as well as the characterisation and detection of organisms (Allman et al., 1992; Fouchet et al., 1993; Porter et al., 1995). Some recent applications have focused on the food industry. They include detection and quantification of food borne micro-organisms and microbial contaminants in food (Ueckert et al., 1995; Jericho et al., 1996; Tortorello et al., 1998). Flow cytometry has proven to be a highly sensitive method for counting cell suspensions as dilute as $10^2$ cells/ml (Pinder et al., 1990; Sørensen and Jakobsen, 1997).

There are a number of food borne micro-organisms which can exist in different morphological forms (Frazier and Westhoff, 1988). In this study, *Brochothrix thermosphacta* was used as the test micro-organism. It is a food spoilage bacterium which causes flavour deterioration of meat and meat products (McLean and Sulzbacher, 1953; Borch et al., 1996). Since this bacterium occurs both as coccobacilli as well as long rods and chains, which form during growth (Davidson et al., 1968), it is difficult to quantify cell numbers. The purpose of this work was to compare different counting methods and evaluate the efficacy of flow cytometry for counting different morphological forms.

## 2. Materials and methods

### 2.1. Organisms and medium

*Brochothrix thermosphacta* MR 165 (NCFB 2891) was maintained on Nutrient Agar (NA, Oxoid, Basingstoke, UK) slopes at 5°C. Working cultures were grown at 25°C for 18 h on Brain Heart Infusion Agar (BHIA, Oxoid) and stored at 5°C. The growth medium, All Purpose Tryptone (APT) (Dainty and Hibbard, 1980), contained the following (g/l in distilled water): tryptone (Oxoid) 10.0; yeast extract (Oxoid) 5.0; NaCl 5.0; $K_2HPO_4$ 5.0; $Na_3$citrate.$2H_2O$ 4.0; $MgSO_4.7H_2O$ 0.8; $FeSO_4.7H_2O$ 0.04. The pH was adjusted to 7.0 with HCl or NaOH. Glucose solution was autoclaved separately and added to give a final concentration of 1% (w/v). The growth medium was inoculated by adding six loopfuls of bacteria from a BHIA plate to 100 ml of growth medium.

### 2.2. Growth experiments

All experiments were carried out at a constant temperature of 25°C and cultures were shaken continuously at 200 rpm. The initial concentration of bacteria (as indicated by both FC and manual counts) was in the range $1 \times 10^5$–$1 \times 10^8$ cells/ml. Growth data were obtained intermittently and comparisons were made between the different methods, i.e. turbidimetry, dry weight, plate counts, flow cytometry and manual counts by microscope.

Optical densities of suitably diluted (if necessary, diluted to an optical density value less than 1.0)

bacterial cultures were determined in duplicate at 610 nm (Series 1000, Cecil Instruments, Cambridge, UK). For dry weight determination, a 10-ml sample was filtered through a 0.02-μm filter paper, placed on a watch glass and then dried at 105°C to a constant weight. For the plate count method, samples were diluted in normal saline diluent (0.9% NaCl), after which 0.1-ml aliquots were spread in duplicate on NA (Oxoid) plates. Viable numbers were estimated from colony counts after an incubation time of 24 h at 25°C. Manual counts (direct microscopic count) were conducted by diluting samples to $10^6$–$10^7$ cells/ml. Equal volumes (100 μl) of sample and stain (0.1%, v/v Methylene Blue) were mixed and incubated for 2 min. The mixed solution was placed in a haemacytometer (improved Nebauer type) and examined under $\times 400$ magnification. Four replicate samples were measured in each case. Where cells existed in chains, the individual cells in a chain were counted.

### 2.3. Flow cytometric analysis

The samples were diluted where necessary with sterile, 0.22 μm-filtered distilled water to within the range $1–3 \times 10^6$ cells/ml. For staining, TO-PRO-3 iodide (Molecular Probes, Oregon, USA) was used (as described by the manufacturer's protocol). This dye is a nucleic acid stain that penetrates permeable (dead) cell membranes. Samples were mixed by agitation and analysed immediately on the flow cytometer (Microcyte Flow Cytometer Model 8000, Aber Instruments, Aberystwyth, UK). This portable flow cytometer (FC) is equipped with a diode laser as the light source and two diode state photo detectors: one for forward light scatter and one for fluorescence. Fluorescence was measured by exciting at 635 nm and measuring emission in the range 650–800 nm. Forward light scatter was used to count the total number of cells (live and dead). The sample uptake rate was 30 μl/min with a total sample analysis time of approximately 10 s. Ten replicate samples were measured.

### 2.4. Percentage viability

Both TO-PRO-3 iodide and Methylene Blue stain dead cells. The percentage viability was determined using the following simple formula: $100 - [(\text{dead cell numbers/total cell numbers}) \times 100]$.

### 2.5. Morphology and size determination

Samples from APT liquid medium were taken at intervals and examined both by microscopy and coulter counter assay (Coulter Multisizer II, Coulter Electronics Limited, Luton, UK). For the former, samples were diluted and transferred to a chamber of 20-μm depth and covered with a cover slip prior to placing onto a microscope stage. Images of bacteria were captured and displayed on a computer using an Image Analysis system (Leica Ltd, Cambridge, UK). For coulter counter analysis, the samples were diluted in ISOTON II. The numbers and size (based on an equivalent spherical diameter) of bacteria suspended in solution were determined automatically.

## 3. Results

### 3.1. Bacterial morphology

Images of *B. thermosphacta* taken during the different stages of growth at 25°C can be seen in Fig. 1. Significant variations were observed in morphology, from coccobacilli to the chain form, which were associated with the different growth phases. At 1–3 h, the culture is in the lag phase (Fig. 1a–d), from 3–9 h growth is exponential (Fig. 1d–g) and after 9 h the culture is in the stationary phase (Fig. 1g–j). When first subcultured to a new medium, the cells comprised of coccobacilli and short rods. During the lag phase, the rods increased in length. When fission occurred, the cells remained associated, thus long chains were observed. Single long rods could be seen at around the mid-exponential phase (Fig. 1f). Cell division continued during this phase, with bacterial chains not increasing further in length. The morphology at the end of the exponential phase exhibited short rods in a chain, and then broke-up into single cells or rods in pairs during the stationary phase. Similar changes in bacterial morphology were also observed when cultures were grown at 5, 10 or 20°C (data not shown).

A coulter counter was used to study the size distribution of bacteria and the results can be seen in

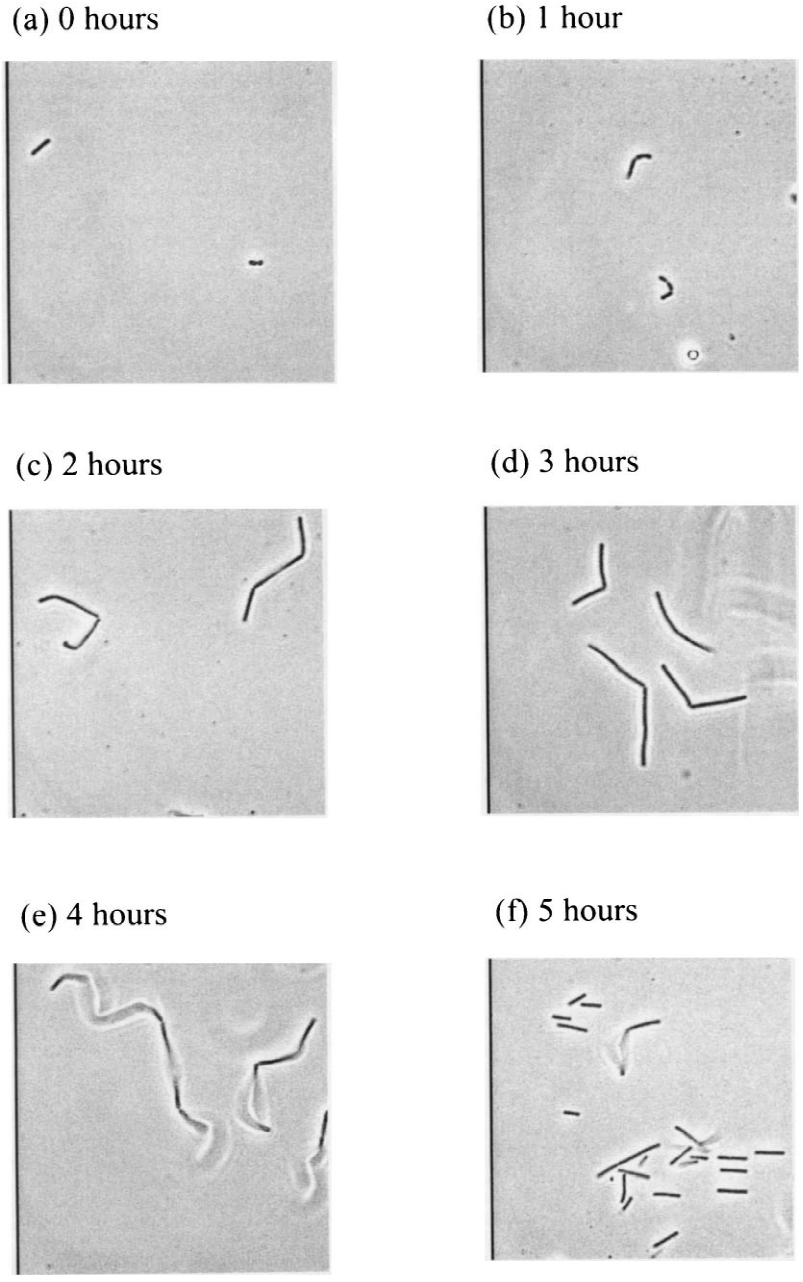


Fig. 1. Morphology of *Brochothrix thermosphacta* during different stages of its growth: (a) 0 h, (b) 1 h, (c) 2 h, (d) 3 h, (e) 4 h, (f) 5 h, (g) 7.5 h, (h) 9 h, (i) 11.5 h, (j) 29 h (×1000 magnification).

Fig. 2. Coulter counter analysis indicates that individual bacteria or bacterial chains have an equivalent spherical diameter in the range 0.7–3 μm. The mean equivalent spherical diameter, in general, decreased with the age of the culture from 1.81 μm at 3 h to 1.28 μm at 24 h. From Fig. 1, assuming that the change in bacterial cell width is negligible, this represents a decrease in bacterial chain length by a factor of almost three over the same period of time.

The observed morphologies are similar to those reported by Davidson et al. (1968) for *B. thermosphacta* growing on nutrient agar. These authors

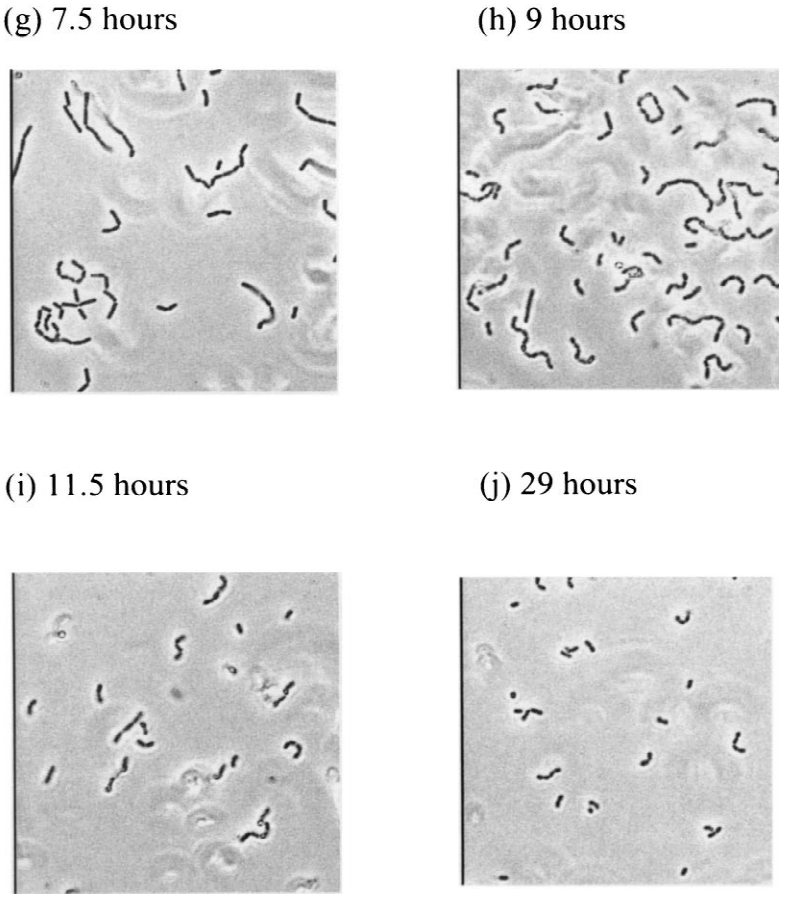


Fig. 1. (*continued*)

demonstrated the existence of individual cells in chains with fully formed crosswalls along the length of the chains. In the present study, attempts were made to separate the individual cells in chains by sonication in order to simplify enumeration of this micro-organism. The sample was treated by diluting in phosphate buffer mixed with 0.05% Tween 80 and then being held in a sonicator bath for up to 30 s. The results showed that there was no difference in morphology before and after such treatment and, therefore, separation could not be achieved under these conditions.

### *3.2. Comparison of counting methods*

The principle purpose of this study on *Brochothrix thermosphacta* was to compare data from the four different enumeration techniques. Samples were taken from all the different phases of growth. Turbidimetric measurements were chosen as the standard in each experiment and were performed simultaneously with the other techniques.

The relationship between optical density and cell count can be seen in Fig. 3 for the three different cell enumeration techniques. All three techniques: FC, manual counts and plate counts were found to give linear relationships to optical density over a narrow range of $10^7$–$10^9$ cells/ml. For example, when viable cells were counted by FC or manual counts, a linear relationship with an $R^2$ value of 0.94 is obtained over this range. Beyond a cell concentration of $10^9$ cells/ml, there is relatively little increase in optical density with cell number for all three techniques.

The FC method was also compared with the manual count method and the numbers counted by the former were marginally higher than the latter as can be seen in Fig. 4. When the line of equivalence

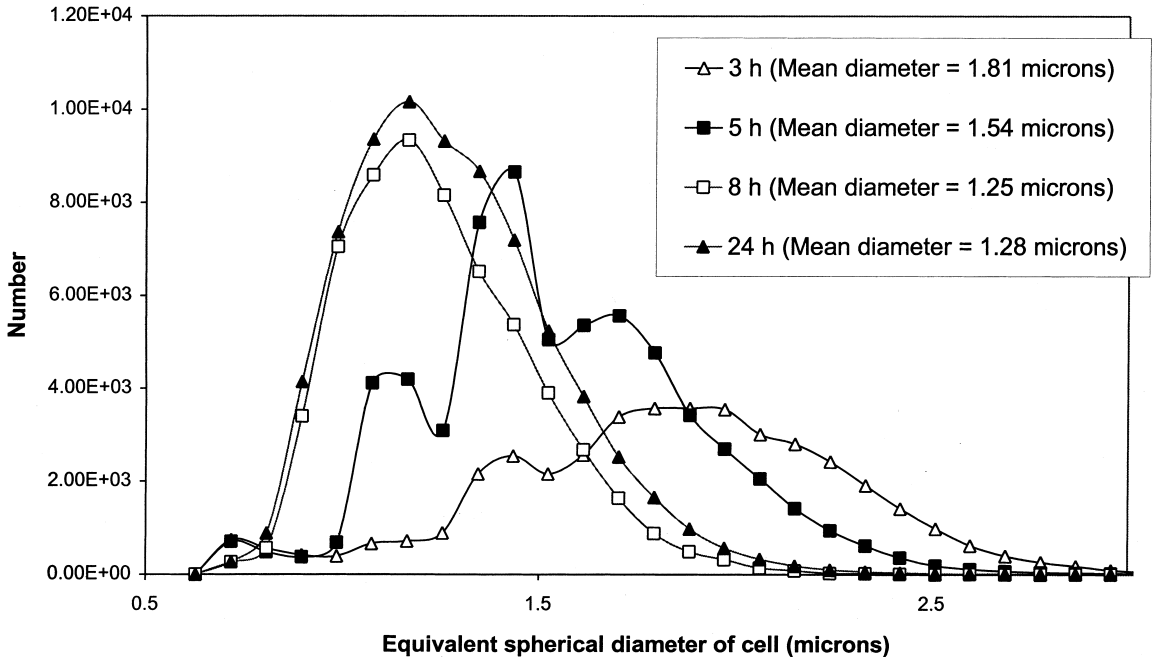


Fig. 2. Cell size distribution as measured by coulter counter.

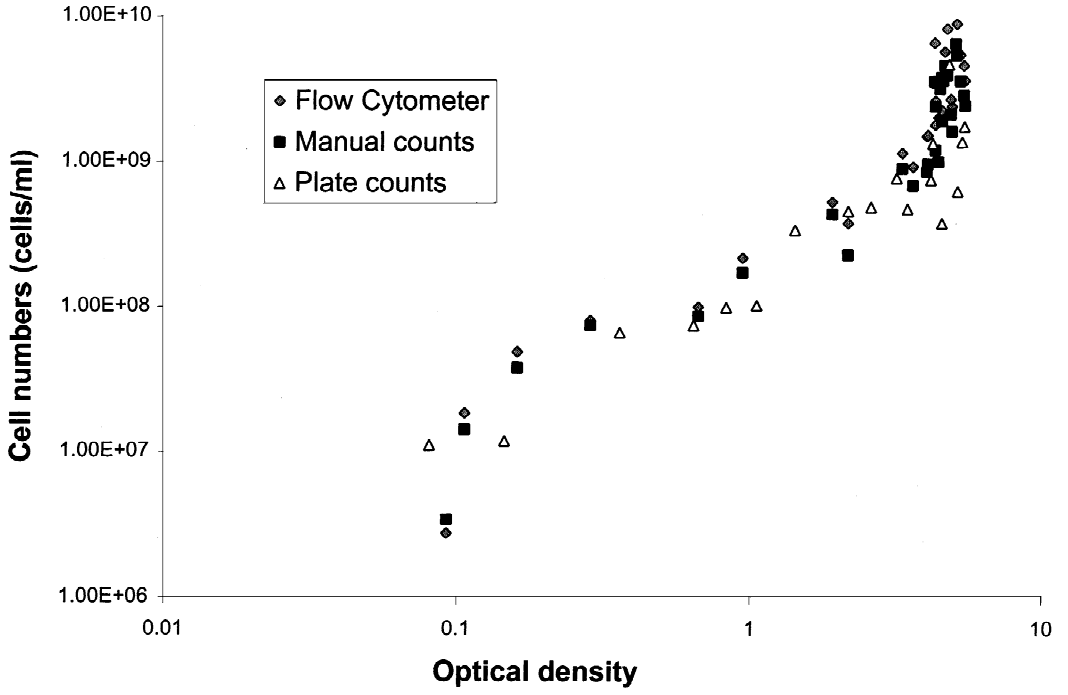


Fig. 3. The relationship between viable cell count and optical density using different enumeration techniques.

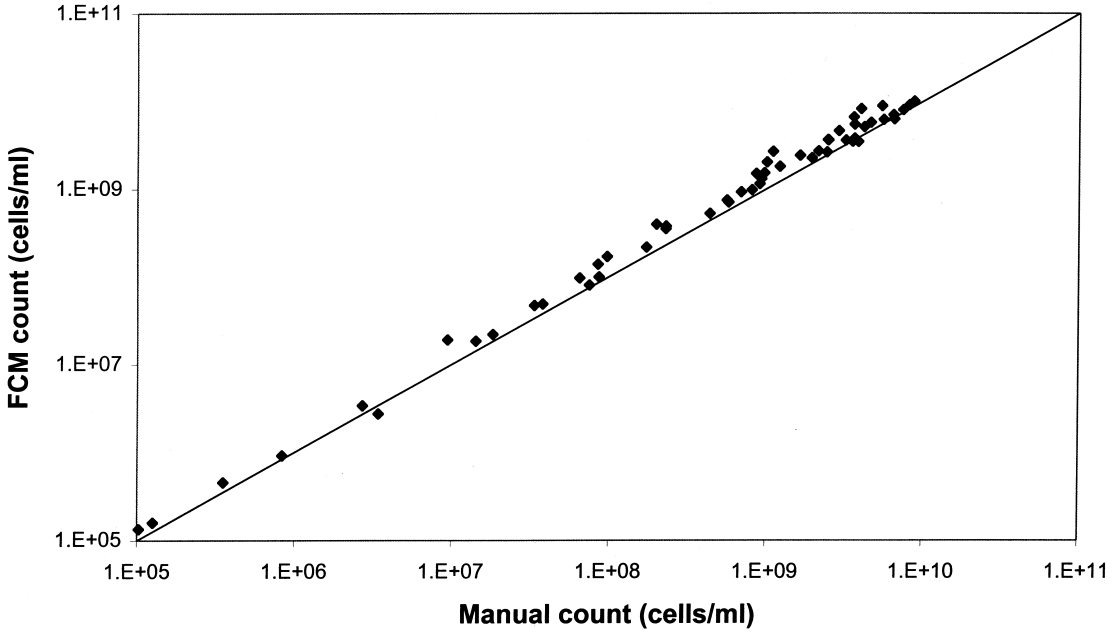


Fig. 4. The relationship between viable cells counted by flow cytometry and manual counts.

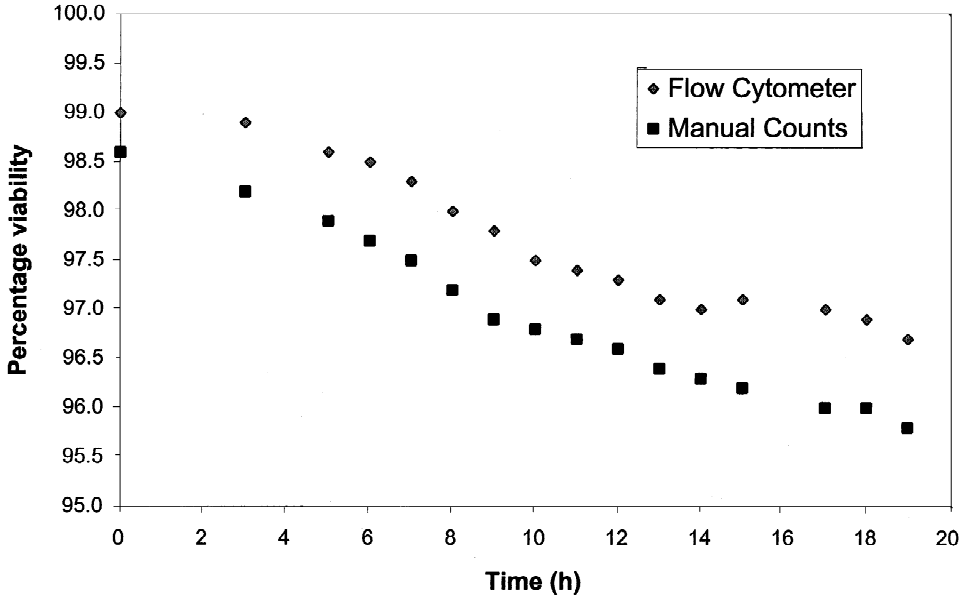


Fig. 5. Typical graph of the percentage viability of *Brochothrix thermosphacta* when counted by flow cytometry and manual counts.

Table 1
The percentage standard error from flow cytometry and manual count methods

| Data set no. | Percentage standard error | | | |
|---|---|---|---|---|
| | Flow cytometry | | Manual | |
| | Total numbers | Viability | Total numbers | Viability |
| 1 | 5.9 | 0.34 | 7.5 | 1.06 |
| 2 | 3.8 | 0.10 | 8.2 | 0.76 |
| 3 | 3.2 | 0.11 | 4.8 | 0.63 |
| 4 | 3.3 | 0.11 | 8.1 | 0.64 |
| 5 | 3.9 | 0.17 | 8.3 | 0.59 |

was drawn across this graph, a linear relationship to experimental data points was observed with an excellent correlation coefficient ($R^2 = 0.99$). Additionally, a linear relationship was attained over a far wider range, of approximately $10^5$–$10^{10}$ cells/ml. On the basis of these results, FC and manual counts can be used over a wider range of cell concentrations than turbidimetry, by two orders of magnitude below and one above.

Fig. 5 shows the percentage viability from FC and manual counts from a typical experiment. The FC technique gave a higher value for percentage viability than manual counts. The counts determined by FC and manual counts were conducted using ten and four replicates, respectively, to confirm the accuracy. The percentage standard errors of manual counts from every data set are higher than those from FC (Table 1). This is because FC counts all cells in a constant sample volume automatically but manual counts depend on human decisions to define cell numbers. Furthermore, the total number of cells counted using flow cytometry will typically run into several tens of thousands, several orders of magnitude higher than the number obtained using manual counts. Differences in the percentage viability recorded between the different techniques may also be due to the different stains used. Both stains, TO-PRO-3 iodide and Methylene Blue can freely enter

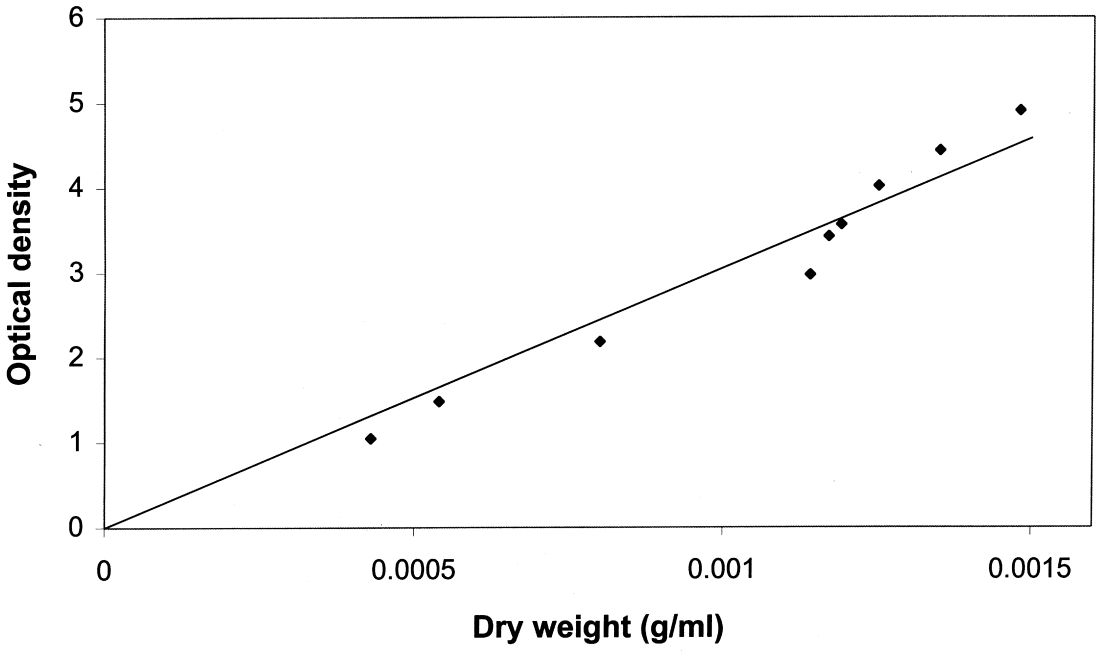


Fig. 6. The relationship between dry weight and optical density.

permeable (dead) cells (Boon and Drijver, 1986). Although, it is known that Methylene Blue can have a toxic effect on cells, reports suggest that this occurs over a time scale of several hours (Boon and Drijver, 1986). In the present study, cells were only exposed for a few minutes to the stain before counting. A possible reason for the discrepancy in results obtained when using the different stains is that TO-PRO-3 iodide may not stain all dead cells. Overall, however, both techniques show reasonably good agreement, giving high percentage viabilities for the bacterial culture.

Fig. 6 shows good agreement between optical density and dry weight readings which can be fitted using a suitable linear relationship ($R^2 = 0.95$). Hence, regardless of cell morphology, turbidimetry appears to provide a good description of the total increase in cell mass during growth, provided there are at least $10^7$ cells/ml.

### 3.3. Bacterial growth

As has already been pointed out, the morphology of the bacterium changes during growth. It is important to know how the application of different enumeration techniques affects the calculated growth related parameters. Fig. 7 shows plots of the logarithmic growth ratio ($\ln N/N_0$) against time as measured using FC, and manual and plate counts, where $N$ is the bacterial number density and $N_0$ is the initial number density. The data was analysed using a paired sample hypothesis statistical analysis method. Using a 95% confidence interval, no statistically significant difference could be found between the data presented for FC and manual counts. However, comparisons between plate counts and manual counts or plate counts and FC did find a statistically significant difference.

The growth of the bacteria after 3 h (at the end of the lag phase, when the bacteria had formed chains) and after 5 h (at the mid-exponential phase, when the bacteria had broken-up into single cells) was compared. Such data are shown in Table 2 in the form of a logarithmic growth ratio for the different enumeration techniques. Furthermore, growth curves were fitted using the model of Baranyi et al. (1995) to calculate growth rate and lag time. This model has been shown to provide a superior description of the bacterial growth curve when compared to more traditionally used sigmoidal functions such as the Gompertz model (Baranyi et al., 1993; Baranyi et al., 1995). The calculated growth related parameters from these curves are shown in Table 3 as well as their corresponding standard errors. The data were

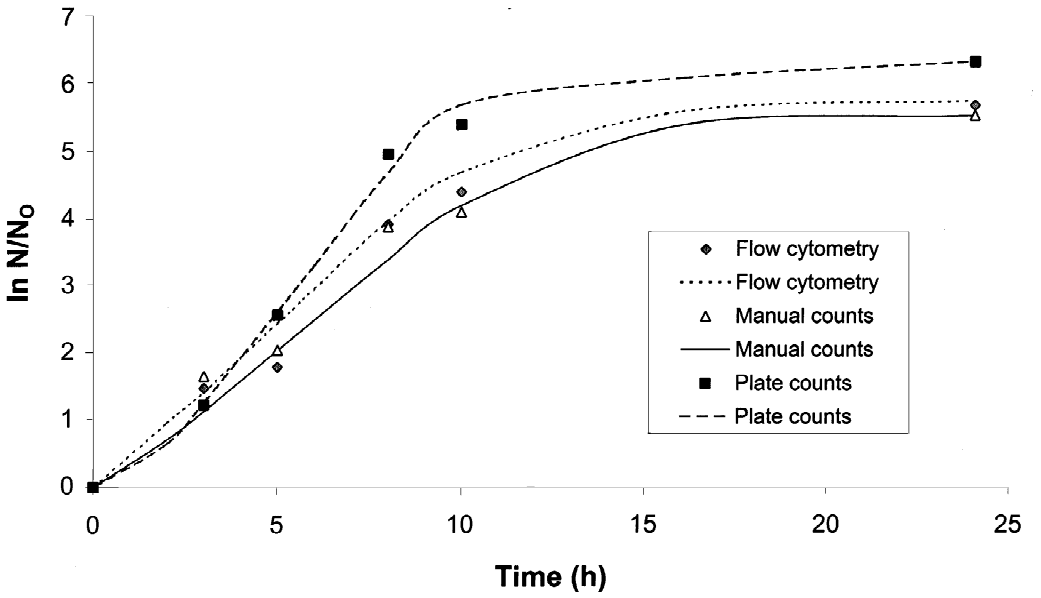


Fig. 7. The growth of viable cells counted using different methods (curves fitted using the model of Baranyi et al., 1995).

Table 2
A comparison of counting methods at difference phases of growth

| Methods | | $Ln(OD/OD_0)$ or $ln(N/N_0)$ | |
|---|---|---|---|
| | | End of lag phase | Mid-exponential phase |
| *Turbidimetry* | | 1.50 | 2.99 |
| *Flow cytometry* | Total cell | 1.48 | 2.51 |
| | Viable cell | 1.48 | 2.50 |
| *Manual count* | Total cell | 1.66 | 2.39 |
| | Viable cell | 1.66 | 2.41 |
| *Plate count* | Viable cell | 1.22 | 2.56 |

Table 3
Comparison of growth related parameter calculated from different counting methods

| Methods | Growth rate ($h^{-1}$) | Lag time (h) |
|---|---|---|
| Turbidimetry | 0.86 (3.2)[a] | 1.26 (3.8) |
| Flow cytometry | 0.58 (3.3) | 1.32 (1.1) |
| Manual count | 0.52 (1.5) | 1.07 (2.6) |
| Plate count | 0.77 (4.2) | 1.65 (6.4) |

[a] Numbers in brackets indicate the percentage standard deviation.

analysed using a students' *t*-test. The growth rates, as calculated using data from manual counts, were found to be significantly different (using a 95% confidence interval) to the other three techniques.

Turbidimetry, FC and manual counts gave a similar value for logarithmic growth ratio after 3 h, whereas plate counts gave a slightly lower value. This highlights the principal disadvantage of the plate count method, i.e. that a single colony may arise from more than one cell. Consequently, the plate count method yields the longest calculated lag time. At the mid-exponential phase (approximately 5 h), bacterial chains break-up into single cells. This results in a sudden increase in cell numbers counted using this technique, which hence produces a calculated growth rate higher than that for FC or manual counts. At 5 h, owing to the existence of predominantly single cells, cell numbers counted using plate counts were closer to that enumerated by FC or manual counts.

At the mid-exponential phase of growth, turbidimetry appears to over estimate cell numbers. Furthermore, the growth rate is somewhat higher than that calculated using the other techniques. It has been shown that suspension optical density increases in proportion to dry weight. The higher growth rate value calculated can be accounted for if one considers that during the early exponential phase (Fig. 1c–e), cells may increase not only in numbers but size, hence producing a logarithmic growth ratio higher than that estimated by the use of the other techniques.

The manual count method overcomes the disadvantages of plate counts or turbidimetry as it is possible to visualise each of the individual cells under the microscope. Table 2 shows that at 3 and 5 h of growth, both FC and manual counts give similar values for logarithmic growth ratio. Furthermore, the growth related parameters calculated using these two techniques were similar. The excellent all round agreement attained between the use of these two techniques suggests that FC is capable of counting the individual cells in a chain.

## 4. Discussion

Different counting methods were used to count the filamentous bacteria, *Brochothrix thermosphacta*. In liquid culture, the morphology of this organism changes significantly from coccobacilli to long rod and forms chains during exponential growth and short rods during the stationary phase of growth. Davidson et al. (1968) reported similar morphological forms for the growth of *B. thermosphacta* on nutrient agar. At the centre of the colony, where it can be assumed that cells are no longer dividing, cells were present as coccobacilli, in short chains or irregular clusters. At the periphery, where cells may

be actively dividing, cells occurred as long rods, present in chains.

The turbidimetric, plate count, FC, manual count and dry weight methods have been compared for the enumeration of *B. thermosphacta*. The turbidimetric method gave a linear relationship only in the range of $10^7$–$10^9$ cells/ml when compared with plate counts, FC and manual counts. The lower sensitivity of turbidimetry from this study is consistent with other studies which show this method to be unable to detect cell concentrations less than about $10^7$ cells/ml (for example, Dalgaard et al., 1994). Towards the end of the exponential phase of growth (cell concentrations higher than $10^9$ cells/ml), further morphological changes occur, with a subsequent decrease in cell size. The decrease in cell size towards the end of the exponential phase of growth explains the plateau region, which can be seen in Fig. 3, as increases in cell numbers fail to produce any significant increase in turbidimetry. Hudson and Mott (1994) analysed the growth of *Pseudomonas fragi* using turbidimetry and plate counts to obtain bacterial growth related parameters. The failure of these two techniques to agree was ascribed to the fact that cell size (instead of numbers) increased during the lag phase, which consequently increased optical density, thus making it difficult to enumerate cells using turbidimetric readings. The same effect was believed to be responsible for an over-estimation of the growth rate of *B. thermosphacta* using turbidimetry. Nonetheless, turbidimetry is commonly used in studies of bacterial growth. Results from this study indicate a measure of caution should be exercised with regards to the accuracy of the data so attained.

Many previous studies have compared FC and plate counts as analytical tools. Pinder et al. (1990) conducted such studies using five orders of magnitude of cell concentration ($10^3$–$10^7$ cells/ml), for four different bacteria of differing sizes and shapes. Excellent agreement was attained across the entire range of cell concentrations. Laplace-Builhe et al. (1993) enumerated bacteria on spinach products, and Sørensen and Jakobsen (1997) studied the yeast *Debaryomyces hansenii*. In both studies, good linear correlations could be attained between FC and plate counts. The present study indicates that plate counts are not suited for the enumeration of bacteria such as *B. thermosphacta* which form cells in chains. Bacterial cells from a 3 h culture were examined under the microscope and the average number of cells per chain determined (approximately 2000 cells were counted). Chains were found to possess an average of 2.46 cells, suggesting that the plate count technique will significantly undercount cell numbers, thereby highlighting a possible shortcoming of this traditionally used technique.

The efficacy of FC as an analytical tool was assessed by comparing it to manual counts, which enables one to identify the individual cells in a chain. Excellent agreement was achieved in cell counts between these two techniques. A similar comparison was made by Laplace-Builhé et al. (1993) when examining the growth of Lactobacillus in skim milk medium. In that particular study, FC somewhat undercounted when compared to manual counts. The Lactobacillus samples were sonicated prior to analysis to break bacterial chains. The fact that FC undercounted was attributed to the fact that some bacteria still remained in chains, with the FC only being capable of counting a chain as a single cell. Those results differ from those presented here, as our results clearly show that FC is capable of counting individual cells in a chain. This discrepancy in results between their study and this one was believed to be because the passage of *B. thermosphacta* through the FC resulted in a break-up of bacterial chains into single cells. This was tested experimentally by passing broth (undiluted and undyed), which had been cultured for 8 h, through the FC and collecting the exiting fluid. Fig. 8 shows pictures of bacteria before and after passage through the FC, clearly demonstrating that cells in chains are broken-up into single cell units. To the authors' knowledge, no previous report has been made in the literature of the breakage of bacterial chains into single cells. Similar results were observed when bacteria of differing culture ages were passed through the flow cytometer.

The mechanism by which bacterial chains are broken by passage through this FC is unknown and merits further investigation. However, one possible explanation is the high acceleration that bacterial chains are exposed to as they undergo hydrodynamic focusing in the flow cell of the FC which may pull the chains apart. Hydrodynamic focusing is the process by which a carrier fluid is used to centrally align the sample fluid in a narrow tube prior to

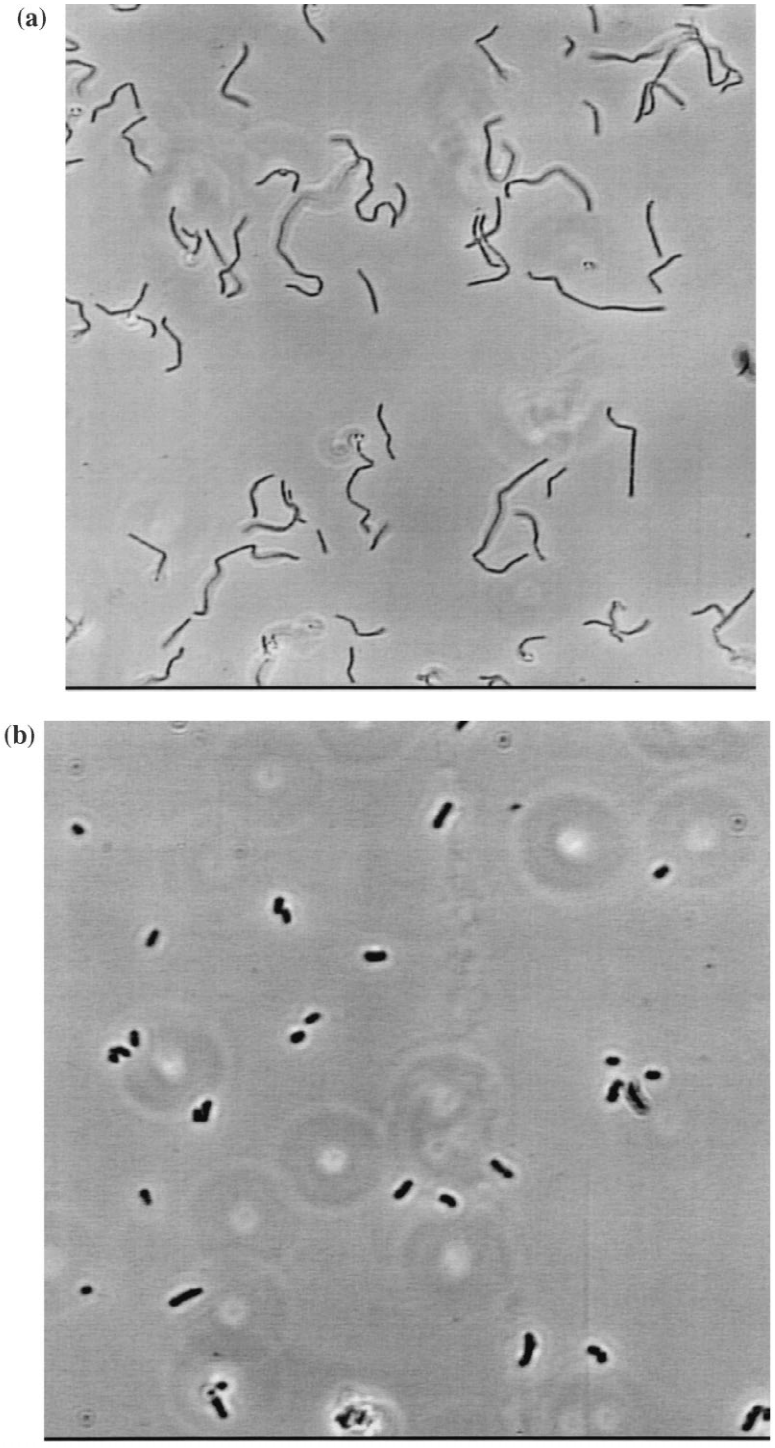


Fig. 8. Cells of *Brochothrix thermosphacta* cultivated for 8 h: (a) before passage through FCM (×400 magnification), (b) immediately after passage through FCM (×1000 magnification).

passing through a laser beam. Details of this process can be found in the literature (Shapiro, 1988). Further experimental work will involve the use of micromanipulation rigs which can measure the tensile strength required to pull bacterial chains apart (Thomas and Zhang, 1998). The forces thereby measured can be compared to the acceleration force acting on the bacterial chains in the flow cell which can be determined via the use of computational fluid dynamic techniques.

Techniques capable of accurately quantifying bacterial numbers throughout the entire range of growth phases under conditions of differing morphologies are an asset. Such data is extremely useful for constructing models of microbial growth for use in predicting food processing or storage (McClure et al., 1994). The portable flow cytometer used in this study (the Microcyte) has demonstrated itself to be capable of fulfilling such a task for a bacterium (*Brochothrix thermosphacta*) with complex morphology. Furthermore, flow cytometry has the advantage that it is a rapid technique with high numerical resolution, capable of assessing cell physical viability and measuring the heterogeneity within a population (Al-Rubeai and Emery, 1996). These features make it attractive as a technique for usage in the food industry for the rapid on-line quantification of microbial flora. However, there are disadvantages that may limit the applicability of this technique. Food samples are likely to need pre-treatment (e.g. homogenisation/filtration) prior to injecting into the FC to prevent blockage of the flow cell. Furthermore, the sample size used is relatively small which makes the detection of trace contamination in large process volumes difficult.

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