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Determination of cell number and size of a population of *Pseudomonas fluorescens* by image analysis

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Microscopic observations of a population of *Pseudomonas fluorescens* were digitised by a frame grabber and an appropriate threshold was chosen to extract the objects from the background. Fully grown, single bacteria were normally distributed around two mean sizes. Two Gauss functions were fitted by least square analysis to these points and the enumeration of single cells was obtained from the area of each Gauss curve and made for each threshold selected. The number of particles counted was constant over a large range of threshold (80–180) whereas the cell area increased with the threshold installed.

Introduction

There are numerous methods to determine biomass values. Bacterial cell enumeration by microscopic observation and determination of cell mass concentration by dry weight are two examples of off-line methods that are often used. In gravimetric determination, the need to dry and weight biomass makes this procedure very time consuming and also susceptible to errors. Microscopic observation usually gives accurate information but a large number of cells must be counted in order to obtain statistically significant data, which makes this task laborious and time consuming (Sonnleitner *et al.*, 1992).

Computer-aided automatic enumeration and characterisation by image analysis overcomes the above described problems, minimising human operation and providing accurate results in a rather limited interval of time (Pons *et al.*, 1992, 1993; Vaija *et al.*, 1995; Zalewski *et al.*, 1994). In order to enable the computer to analyse grabbed images, a proper threshold has to be chosen dividing the image in background pixels and cells (Otsu, 1979).

The aim of this work was to characterise a heterogeneous population of *Pseudomonas fluorescens in situ* by image analysis. Based on the cell size distribution, a methodology was developed to determine the cell size and number simultaneously.

Materials and methods

Bacteria sampling

Pseudomonas fluorescens was grown in a culture medium (0.5% (w/v) glucose, 0.25% (w/v) peptone and 0.125% (p/v) yeast extract) during 8 hours. The biomass was harvested by centrifugation, washed, resuspended in distilled water and 50 µl was fixed on a glass slide and coloured with Methylene Blue to obtain a good contrast between cells and background.

The microscopic observations were carried out with an inverted phase contrast microscope, using a phase contrast 40× objective, and a TV relay lens 1X (Nikon-Japan) adapted to the video camera. 25 microscope observations were made for 4 samples, giving a total study of 100 images.

Image analysis and automated enumeration

The microscopic image was received by a CCD video camera (Sony AVC D5CE) and the image was digitised by a frame grabber (DT2851 Data translation Inc.) installed in a 486 DX4 100 MHZ personal computer. The grabbed images consist of a 512 × 512 pixels array, each pixel has a grey-level intensity value ranging from 0 (black) to 255 (white).

At the beginning of the experiment, an out-of-focus image was grabbed and stored on the hard disk of the computer. From two in-focus grabbed images the

background was subtracted in order to remove contaminations on lens and camera and to obtain an uniform background. The resultant two images were multiplied, reducing noise and enhancing the contrast of the objects in the final image (Meinders *et al.*, 1992).

Since the computer can only analyse binary images, an adequate threshold had to be chosen. The enumeration was made on the final images as a function of the threshold installed in a range of threshold between 1 and 255. The threshold was optimized by Otsu's method (Otsu, 1979)

Data analysis

The determination of bacterial number was made based on the cell size distribution of cells. Assuming a normal distribution of bacteria area around a mean size, the cell size distribution can be described by a Gauss function:

$$K.e^{-((x-\bar{a})/\sigma)^2} \quad (1)$$

in which K is a normalisation factor and σ the standard deviation (of the size).

The integration of Eq. (1) results in the number of cells, distributed around a mean size a.

$$n = \int K.e^{-((x-\bar{a})/\sigma)^2} = \pi.k.\bar{a} \quad (2)$$

The above described procedure can also be used for the determination of the number of single cells (n_s), doublets (n_d), triplets (n_t) and multiplets (n_m). The total number of objects (n_{obj}), can simply be obtained by counting the number of objects.

Since bacteria can aggregate forming doublets, triplets and multiplets of bacterial cells, the number of total cells (n_{bt}) do not corresponds to the number of total objects (n_{obj}) counted. Once the mean size of a single cell is known, the total number of bacterial cells, (n_{bt}), can be obtained from:

$$n_{bt} = \sum_{i=1}^{n} \frac{a}{\bar{a}_s} n_i \quad (3)$$

where n_i is the number of objects (i) detected, having a area a_i and a_s is the mean area of a single bacteria. The above described procedure was repeated for all values of threshold installed.

Results and discussion

Pseudomonas fluorescens are rod shaped cells but they can flip over, adopting both standing (circle shaped) and lying positions (rod shaped). Figure 1 represents the area distribution of *Pseudomonas fluorescens* at the optimum calculated threshold of 113, where two peaks and a long tail can be clearly seen: the first peak corresponds to circle shaped cells (standing cells), the second peak to rod shaped cells (lying cells) and the long tail to doublets, triplets and higher order multiplets of bacterial cells. The data points show normal distributions around two mean values, hence two Gauss functions (Eq. 1) were least square fitted. Since the mean area of both curves coincide with the peaks of the data points, the noise and the higher order multiplets have a minor influence on the Gauss curves.

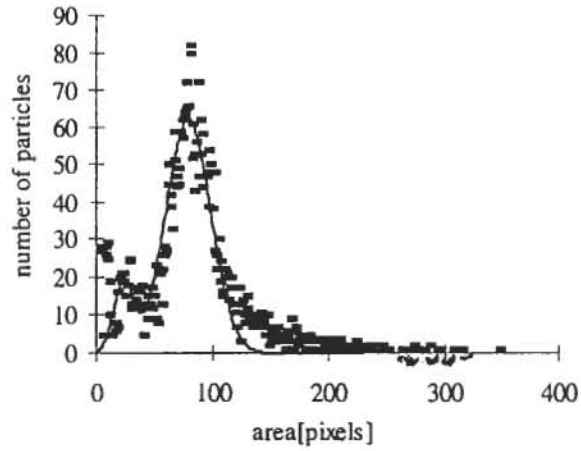

Figure 1 Cell size distribution of *Pseudomonas fluorescens* at an optimum threshold of 113.

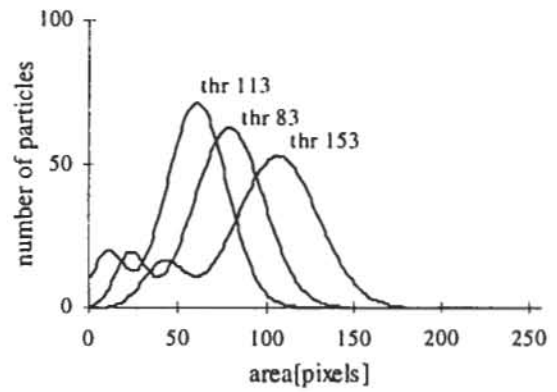

Figure 2 Cell size distribution of *Pseudomonas fluorescens* at thresholds 83, 113 and 153.

Figure 2 shows the area distribution of *Pseudomonas fluorescens* at thresholds 83, 113 and 153. The threshold has a strong influence on the mean size of bacteria, since as the threshold increases the distribution is displaced to the right, moreover, at threshold 83 not all standing cells were counted.

Due to the good correspondence between the Gauss function and the data points (Fig. 1) the mean projected area of a single bacteria could be found. The mean area increases as function of the threshold (Fig. 3). This is due to the fact that the transition between objects (black) and background (white) is continuous, i.e. as threshold increases more pixels are included in the border of the black object. Though, the multiplication of images was made to improve results (Meinders *et al.*, 1992), the enhancement was still not good enough in order to analyse the area correctly. An improved enhancement procedure should sharpen the transition between background and bacteria. To determine if the obtained area is correct, the area should be constant as a function of the threshold, otherwise the area would depend on the threshold installed.

Another possible explanation for the fact that bacterial area increases with threshold is that as the threshold increases, more multiplets are detected increasing the long tail in Figure 1. This tail tends to pull the curves to the right increasing the mean values. As the standard deviation obtained for both curves had a very small variation with threshold, the width of the curves did not suffer any modification as can be seen clearly in Figure 2. So this had a minor effect on cell enumeration.

Figure 4 shows the number of standing (D) and lying (C) cells as well as the total number of objects (B) and the total number of cells (A) as a function of the threshold installed. From Figure 4, it can be seen that the number of standing and lying cells and the total number of cells are constant while the number of objects increases at low values of threshold and decreases at high values of threshold. Between threshold 83 and 153 the total number of objects (B) remains constant. From the cell size distribution, it was possible to calculate the number of lying and standing cells, by calculating the area of the Gauss curves (Eq. 2). The number of cells aggregated had to be estimated by the mean size of a single cell. Because no Gauss curve could be fitted on the long tail presented in Figure 1. In Figure 4, both number of standing and lying cells is constant. Only at high values of threshold the number of lying bacteria decreases, this is due to the effect of bacteria growing together at high thresholds i.e. two cells that are next to each other are considered as one object. Therefore the appropriate threshold should be lower than the shut of points of the decrease.

Since no double standing cells are formed and the mean area of lying cells is known, the total number of cells can be calculated using Eq. 3. As the total number of cells is constant (Fig. 4A), it can be concluded that enumeration of cells is correct. Moreover the number of objects (single cells, doublets, triplets and multiplets), first increases, remains constant and then decreases. The initial increase is due to the fact that at low threshold not all objects are counted. At high thresholds cells tend to grow together decreasing the number of objects. The fact that both lying and standing cells remains initially constant is a result of the above described two effects. Increasing threshold more single cells are thus more objects are counted, however at the same time singles grow together keeping the number of singles constant whereas the number of objects increases. The appropriate threshold lies in between the range were the number of objects remains constant. In this region, multiplets are really counted as multiplets and single cells as singles. Therefore the optimum threshold lies in between 83 and 153. To make automation possible,

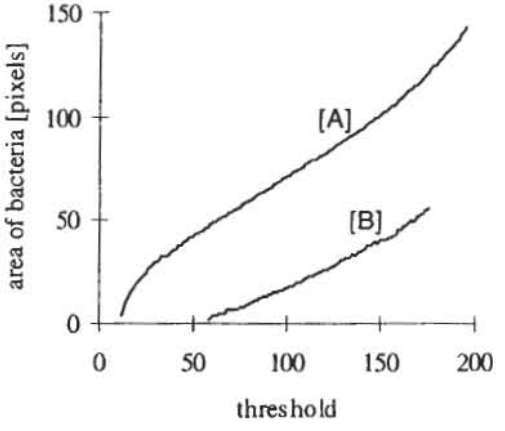

Figure 3 Area of *Pseudomonas fluorescens* as a function of the threshold installed (A, single standing cells; B, single lying cells).

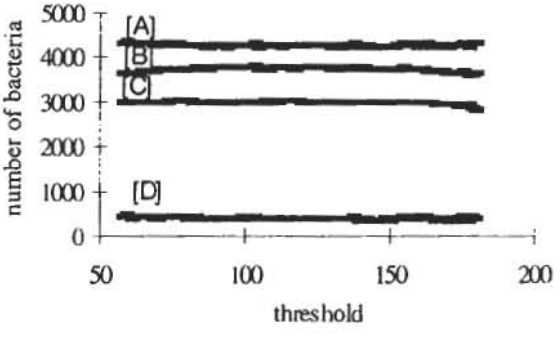

Figure 4 Number of *Pseudomonas fluorescens* counted as a function of the threshold installed (A, total number of cells; B, total number of objects; C, number of lying cells; D, number of standing cells).

Otsu's method was adopted to separate bacteria from background (Otsu, 1979) and the optimum threshold found was 113, almost in the middle of the threshold range.

Conclusions

Image analysis is a useful technique for bacteria enumeration, minimising human operator intervention and providing accurate results in a short interval of time.

The method also proved the ability to compare cell sizes of a heterogeneous population of *Pseudomonas fluorescens*, by determining the cell area distribution. This technique also enables to calculate the size of a single bacteria.

From the area distribution the number of single cells and aggregates can be determined accurately even though the area depends on the threshold chosen.

The selection of an adequate threshold of grey level for extracting the objects from the background is of utmost importance in image analysis. For high and low values of threshold the enumeration depends on the selected value of the threshold. The optimum threshold should be chosen in the range where enumeration is independent of the threshold selected.

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References

Meinders J.M., Van der Mei H.C. & Busscher H.J. (1992). *J. of Microbiol. Meth.* 16: 119–124.

Neu T.R., Van der Mei H.C. & Busscher H.J. (1989). Biofilms associated with health. In: *Biofilm Science and Technology*, L. Melo, eds Vol 223. pp 21–34: Nato ASI Series.

Otsu N. (1979). *IEEE Trans. Syst. Man Cibern.* SMC-9: 62–66.

Pons M., Wagner A., Viver H. & Mark A. (1992). *Biotechnol. Bioeng.* 40: 187–193.

Pons M., Viver H. & Rémy J.F.(1993). *Biotechnol. Bioeng.* 42: 1352–1359.

Sonnleitner B., Locher G. & Fiechter (1992). *J. Biotechnol.* 25: 5–22.

Vaija J., Laugaude A. & Ghommidh (1995). *Ant. van Leewenhoek* 67: 139–149.

Zalewski K., Gotz P. & Buchholz R. (1994). On-line estimation of yeast growing rate using morphological data from image analysis. In: *Adv. Bioprocess Engineering*, E. Galindo and O.T. Ramírez, eds pp 191–195, Netherlands: Kluwer Academic Publishers.

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