# DETERMINATION OF CELL NUMBER AND SIZE OF A POPULATION OF *PSEUDOMONAS FLUORESCENS* BY IMAGE ANALYSIS

J. Azeredo, J. Meinders, R. Oliveira*
Universidade do Minho, Centro de Engenharia Biológica, Braga 4709 codex, Portugal
Tel: 351-(0)53- 604400, Fax: 351-(0)53-604413, email: roliveira@ci.uminho.pt

**Abstract:** Image analysis is a very useful technique for counting and sizing bacteria, minimising human operation and providing accurate results in a short interval of time. Microscopic observations of a population of *Pseudomonas fluorescens* were digitised by a frame grabber and the grabbed images were enhanced by background subtraction and multiplication of two copies. To extract the objects from the background, an appropriate threshold had to be chosen. Full grown single bacterial cells showed to be normally distributed around two mean sizes, one corresponding to standing bacteria and the other to lying bacteria. Two Gauss functions were least square fitted to these data points resulting in the mean area and the standard deviation. The enumeration of single cells was obtained from the area of each gauss curve. It was also possible to determine the number of single bacteria in aggregates, once the mean project area of a single cell is known. The enumeration was made for each threshold selected. The number of particles counted was constant in a large range of threshold, whereas the cell area increases with the threshold installed.

**Key words**: *Pseudomonas fluorescens*, image analysis, enumeration, cell size distribution.

## 1. INTRODUCTION

Biomass quantification and characterisation is very important in different fields of research, such as food industry, medicine and microbiology[3].

There are several methods to determine the amount of biomass. Bacterial cell enumeration by microscope 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 of errors. Microscope 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 very laborious and also time consuming.

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[2,6,7,9]. Moreover computer aided image analysis makes it possible to characterise cells as a function of time[2]. 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.[4]

The aim of this work is 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.

## 2. MATERIAL AND METHODS

### 2.1 Microscopic observations

The microscopic observations were carried out with an inverted phase contrast microscope (Nikon-Japan), using a phase contrast 40x objective, and a TV relay lens 1X (Nikon-Japan) adapted to the video camera. 25 observations of 4 glass slides containing fixed *Pseudomonas fluorescens* were made, giving a total study of 100 images.

### 2.2 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 512x512 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 hard disk of the computer. From two in focus grabbed images the background was subtracted in order to remove contamination 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.[1]

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 also selected automatically using a method described by Otsu.[4]

### 2.3 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 $\bar{a}$, the cell size distribution can be described by a Gauss function:

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

in which k is a normalisation factor and s the standard deviation (of the size).

The integration of Eq. (1) results in the number of cells, distributed around a mean size $\bar{a}$.

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

The above described procedure can also be used for the determination the number of single cells ($n_s$), of doublets ($n_d$), of triplets ($n_t$) and of 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 cells is known, the total number of bacterial cells, ($n_{bt}$), can be obtained from:

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

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

## 3. RESULTS AND DISCUSSION

Microscopic observations of *Pseudomonas fluorescens*, showed that these bacterial cells are rod shaped. However, some circle shaped bacteria were also observed, corresponding to cells seen from its top (standing bacteria). Since bacteria can be found in two different spatial positions (standing and lying), the images studied were considered to show a heterogeneous population.

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 seen clearly: 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 2 shows the area distributions of *Pseudomonas fluorescens* at thresholds 83, 113 and 153. The threshold has high influence on the mean size of bacteria, since as the threshold increases the distribution is displaced to the right, moreover, at threshold 84 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 the 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. Thought, the multiplication of images was made to improve results,[1] however enhancement was still not good enough,

Another possible explanation for the fact that bacterial area increases with the threshold is that as 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.

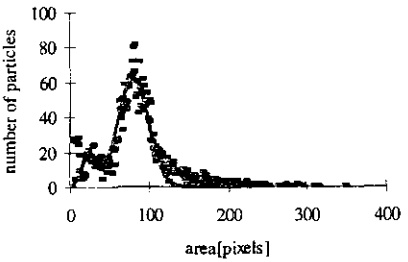


Fig.1: Cell size distribution of *Pseudomonas fluorescens* at an optimum threshold of 113

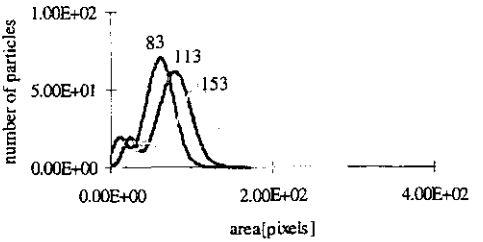


Fig.2: Cell size distribution of *Pseudomonas fluorescens* at thresholds 83, 113 and 153

From the cell size distribution it was possible to extract 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 (Eq. 3), because no Gauss curve could be fitted in 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 on object. Therefore the appropriate threshold should be lower than the shut of points of the decrease and must lie in between the range were the number of objects remains constant (for values of threshold between 83 and 153). In this region multiplets are really counted as multiplets and single cells are really singles.

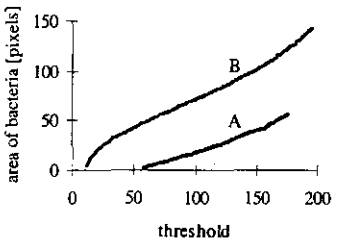


Fig. 3: Area of *Pseudomonas fluorescens* as a function of the threshold installed (A-single standing cells; B-single lying cells)

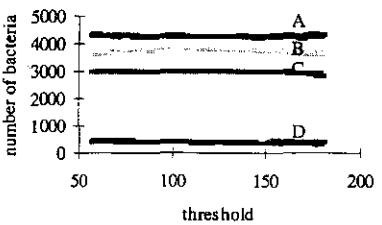


Fig. 4: Number of Pseudomonas fluorescens counted as a function of the threshold installed( A-total number of cells; B-total number of objects; C-number of lying cells; D-number of standing cells)

To make an automation possible, Otsu's method[4] was adopted to separate bacteria from background and the optimum threshold found was 113, almost in the middle of the threshold range

## 4. 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 thought 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.

## REFERENCES


1. Meinders J.M., Van der Mei H.C. & Busscher H.J., In situ enumeration of bacterial adhesion in a parallel plate flow chamber - elimination of in focus flowing bacteria. *J. of Microbiological Methods*, **16** (1992) 119-124.
2. Meinders J.M., Noordmans J. & Busscher H.J., Simultaneous monitoring of the adsorption and desorption of colloidal particles during deposition in a parallel plate flow chamber. *J. of Colloid and Interface Science*, **152** (1992) 265-280.
3. Neu, T, R.,Van der Mei H.C.& Busscher H.J., Biofilms associated with health. *In Biofilm Science and Technology*. Vol. 223. Nato ASI Series, 1989, pp.21-34.
4. Otsu, N., A threshold selection method from gray-level histograms. *IEEE Transactions on systems, man and cibernetics*, **9** (1979) 62-66.
5. Pons, M., Wagner, A., Viver H. & Mark, A., Application of quantitative image analysis to mammalian cell line grown on microcarriers. *Biotechnology and Bioengineering*, **40** (1992) 187-193.
6. Pons, M., Wagner, A., Viver H. & Mark, A., Application of quantitative image analysis to mammalian cell line grown on microcarriers. *Biotechnology and Bioengineering*, **40** (1992) 187-193.
7. Pons, M., Viver H. & Rémy J.F., Morphological characterization of yeasts by image analysis. *Biotechnology and Bioengineering*, **42** (1993) 1352-1359
8. Vaija, J., Laugaude A. & Ghommidh, Evaluation of image analysis and laser granulometry for microbial cell sizing. *Antonie Van Leewenhoek*, **67** (1995) 139-149.
9. Zalewski K., Gotz P. & Buchholz R., On-line estimation of yeast growing rate using morphological data from image analysis. *Adv. Bioprocess Engineering* (1994) 191-195.