DETERMINATION OF CELL NUMBER AND SIZE OF A POPULATION OF *Pseudomonas fluorescens* BY IMAGE ANALYSIS

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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.

Keywords: *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. The traditional methods for biomass quantification, like gravimetric determination or microscope observation are laborious and very time consuming. They require a great number of data to obtain significant statiscal results.

Computer aided automatic enumeration and characterisation by image analysis overcomes these problems, minimising human operation and providing accurate results in a rather limited interval of time[6,7,9]. Moreover computer aided image analysis makes 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[5].

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.

2 MATERIAL AND METHODS

2.1 Bacteria sampling

Pseudomonas fluorescens obtained from Gulbenkian Institute of Science, were grown in a medium containing 5g/l of glucose, 2.5g/l peptone and 1.25g/l yeast extract. After 15 hours of incubation the cells were killed by exposure to microwaves, to allow the measurements with motionless cells. Then the cell aggregates were separated by four times sonication during 4s at 20KHz, 200W. Cells were washed and resuspended in distilled water. 50 µl of this cell suspension 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 (Nikon-Japan), using a phase contrast 40x 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.

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 the 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[5]. In short, the method consisted of separating background and cell pixels into two classes, maximising:

$$\omega_0\omega_1(\mu_1-\mu_0)^2 \qquad (1)$$

where

$$\omega_0=\sum_{i=0}^{k} p_i \quad \omega_1=\sum_{i=k+1}^{255} p_i \qquad (2)$$

and

$$\mu_0=\sum_{i=0}^{k}\frac{i\,p_i}{\omega_0} \quad \mu_1=\sum_{i=k+1}^{255}\frac{i\,p_i}{\omega_1} \qquad (3)$$

and

$$p_i=\frac{n_i}{N} \qquad (4)$$

In which ω_0, ω_1 and μ_0, μ_1 are the zeroth and the first order commulative moments of the histogram of grey values to the k_{th} level respectively, p_i the probability of the number of pixels n_i and N the total number of pixels.

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:

$$K.e^{-((x-\bar{a})/\sigma)^2} \qquad (5)$$

in which k is a normalisation factor and s the standard deviation (of the size). The integration of Eq. (5) results in the number of cells, distributed around a mean size $\bar{a}$.

$$n=\int K.e^{-((x-\bar{a})/\sigma)^2}=\pi.k.\bar{a}, \qquad (6)$$

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.

$$(n_{obj})=\sum y \qquad (7)$$

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:

$$n_{bt}=\sum_{i=1}^{n}\frac{a_i}{\bar{a}_s}n_i \qquad (8)$$

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. In Eq.(8) it is assumed that a doublet has a size of two times the size of a single cell, a triplet the size of three times the size of a single cell and a multiplet a size of m times the size of a single cell. The above described procedure was repeated for all values of threshold installed.

The bacteria size was converted to S.I. units using a bar of known length. The pixel equivalence found was 0.114 μm^2.

The mean area was also obtained with the aid of scanning electronic microscope (SEM) observations of bacteria fixed in plates of PMMA. *Pseudomonas fluorescens* were considered to have the shape of rectangles with hemi-ellipse at each end. The value of the estimated area was obtained through:

$$A=Ll+\pi la \qquad (9)$$

in which L represents the length of the bacteria, l the diameter and a half diameter of the ellipse.

3 RESULTS

Microscopic observations of a population of *Pseudomonas fluorescens* showed rod and circular shaped cells. The circular objects are bacteria seen from their top (standing bacteria) and rod shaped objects are cells seen from its longer size (lying bacteria). Similar observations of alive cells showed that these bacterial cells flip over, adopting lying and standing positions. Doublets of lying bacteria.can also be observed. Since bacteria can be found in two different spatial positions the obtained images 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 can be seen clearly.

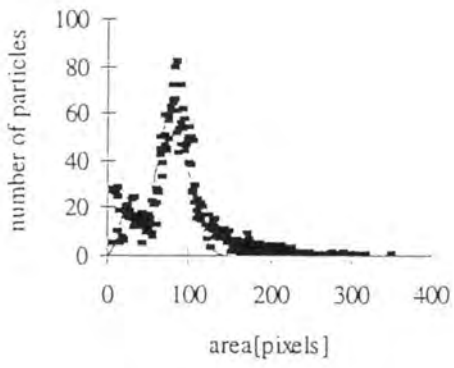

Figure 1 - Cell size distribution of *P. fluorescens* at an optimum treshold of 113.

The data points show normal distributions around two mean values, hence two Gauss functions Eq.(5) were least square fitted. The fitted Gauss functions follow the data points very closely as can be seen from the black line. The standard deviation obtained for these two curves was 12.1% for the first gauss curve and 27.3% for the second curve. Results are presented in Table 1.

Table 1 - Cells number and size calculated at a threshold of 113.

	standing cells	lying cells	total cells	total objects
number	396	3015	4264	3775
size (μm^2)	2.7	9.0		

In Figure 1 very small objects can be seen, corresponding to noise, and a long tail of data points corresponding to doublets, triplets and higher order multiplets. It was not possible to adjust Gauss functions to these higher order multiplets, because they were not a statistically significant number. 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.

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.

Both bacterial areas (lying and standing) increase with the threshold installed. The area of *Pseudomonas fluorescens* determined from the SEM photographs was 2.5μm^2, this value is smaller compared with the result obtained by image analysis presented in Table 1.

The area of the first Gauss curve in Fig. 1 corresponds to the number of standing cells and the area of the second Gauss curve corresponds to the number of lying cells Eq.(6). Since no double or higher order of standing cells were observed, the total number of cells could also be calculated using Eq.(9). In Eq.(9) the area of a single lying cell is used, since only lying cells occur in doublets and multiplets.

From several observations it could 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 remains constant.

4 DISCUSSION

Standing bacteria are present in small number, as can be seen by the height of the two peaks represented in Figure 1. This is due to the fact that the standing position is unstable. It was possible to have a separate distribution of standing and lying cells because doublets or triplets of standing cells were not found and also because, intermediate positions between standing and lying were not found (Figure 1). If cells adopted positions between standing and lying, the deep valley seen in Figure 1 would not be visible, since more objects between the two peaks would be found. Moreover, the good correspondence between the Gauss curves and the data points in Figure 1, shows clearly that the objects positioned in the valley are large standing or small lying cells.

Some authors report on the tendency that *Pseudomonas fluorescens* have to form filaments as a way of surviving, moreover there are no evidences that bacteria adhere side by side[3,8]. The fact that cells do not adhere side by side, was also confirmed by manual observations, since no double standing cells were observed. Since cells adhere top to top, the circle shaped objects observed in Figure 1, could not be only one single, but also two cells on the top of each other. However as this position is very unstable this is not likely to occur.

Least square fitting the data points with a Gauss function shows that the size of bacteria follows a normal distribution (Fig. 1). This fact can be an indication that the bacteria were full grown, since it can be expected that when bacteria are still growing the size distribution would show a long tail to smaller areas. Due to the good correspondence between the Gauss function and the data points the mean projected area of a single bacteria could be found. The mean area increases as a function of the threshold and this is due to the fact that the transition between objects (black) and background (white) is continuous, i.e. as the threshold increases, more pixels are included in the border of the black object. Though, the multiplication of images was made to improve results[1], 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. However, multiplication of two copies with a slight time delay is of high importance in dynamic studies of adsorption and desorption of particles since this procedure yields different grey values for moving and adhering particles[2]

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. So this had a minor effect on cell enumeration.

For the optimum threshold calculated automatically, the value of bacteria area determined was higher than the area calculated by SEM. This difference may be due to the treatments to which cells were submitted for SEM observation. These include the drying of the cells during sample preparation and the insertion in a vacuum chamber. It is expected that both effects can lead to a significant decrease in cell size. Moreover for microscopic observations, cells were ressuspended in distilled water and treated with methylene blue. Due to the low ionic concentration of distilled water, cell volume increases, and methylene blue, colours of blue the borders of the cells making them look like bigger.

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. (6)). The number of cells aggregated had to be estimated by the mean size of a single cell (Eq. (8)), because no Gauss curve could be fitted to the long tail presented in Figure 1. Both numbers of standing cells and lying cells are 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. (8). As the total number of cells is constant, 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 singe cells and 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 are really singles. Therefore the optimum threshold lies in between 83 and 153. To make an automation possible, Otsu's method[5] was adopted to separate bacteria from background (Eq. (1)). The optimum threshold was found to be 113, almost in the middle of the threshold range.

5 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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