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Computer program for quantitative identification of microscope images of cells in biological suspension of selected strains of microorganisms

Abstract. *The paper describes possibilities of automatic identification of Enterococcus faecalis, Candida albicans, Pseudomonas aeruginosa and Saccharomyces cerevisiae cell counts in suspension using a computer program written in Python environment. The software was validated in terms of accuracy of detection of abundance when confronted with traditional counting. The limits of repeatability, reproducibility and expanded uncertainty of measurement were determined for a confidence level of 95%. The software was found to count cells in suspension from images taken under a microscope at 400 times magnification for Candida albicans and Saccharomyces cerevisiae and 1000 times for Enterococcus faecalis and Pseudomonas aeruginosa with an accuracy of 15%.*

Streszczenie. *W pracy opisano możliwości automatycznej identyfikacji liczebności komórek Enterococcus faecalis, Candida albicans, Pseudomonas aeruginosa i Saccharomyces cerevisiae w zawiesinie przy użyciu programu komputerowego napisanego w środowisku Python. Oprogramowanie zostało zwalidowane pod względem dokładności wykrywania liczebności w konfrontacji z tradycyjnym liczeniem. Wyznaczono granice powtarzalności, odtwarzalności i rozszerzonej niepewności pomiaru dla poziomu ufności 95%. Stwierdzono, że oprogramowanie zlicza komórki w zawiesinie ze zdjęć wykonanych pod mikroskopem przy 400-krotnym powiększeniu dla Candida albicans i Saccharomyces cerevisiae oraz 1000-krotnym dla Enterococcus faecalis i Pseudomonas aeruginosa z dokładnością 15%. (**Program komputerowy do ilościowej identyfikacji obrazów mikroskopowych komórek w zawiesinie biologicznej wybranych szczepów mikroorganizmów**)*

Keywords: computer program, quantitative identification, pathogens, microorganisms
Słowa kluczowe: program komputerowy, ilościowa identyfikacja, patogeny, mikroorganizmy

Introduction

The quantitative identification of cells in a biological suspension is a fundamental issue in microbiological and other research, where this information is a marker for generating conclusions and further research proceedings. Several methods are currently used for the identification of microorganisms. One of the basic methods are direct methods based on counting microbial cells under a microscope, e.g.: counting in Thom's chamber, Biuirker's chamber, Breed's method and Direct Epifluoroscent Microscopy (DEM) and Direct Epifluoroscent Filter Technique (DEFT) [1, 2]. The second group consists of indirect methods consisting of measuring the wet weight of microorganisms after centrifugation and washing of cells, measuring the dry weight of yeast bacteria after centrifugation and washing of cells with sterile distilled water and then drying to constant weight at 105 °C, and measuring the volume of microbial sediment after centrifugation in graduated tubes. The third group consists of optical methods based on the relationship between the number of microorganisms in a liquid culture and light transmission, where we distinguish turbidimetric and nephelometric methods. The fourth group are culture methods based on membrane method, dilution method, plate method and spiral culture method. Other methods used for the detection of microorganisms are impedimetry, cytometry and bioluminescence. The reduction of testing time, reduction of absorbance work, ease of sample execution, obtaining more precise results and replacing their subjective evaluation and counting of microorganisms by human beings, possibility of data archiving and analysis makes that rapid methods for microbial quantification should be used for testing routinely performed in pharmaceutical, food and cosmetic industries [3]. Gray-level image identification is well known and used, for example, for the identification of organic matter shape parameters [4], while more sophisticated image analysis is machine vision systems used in engineering, biomedicine, agriculture and forestry [5-13]. Practice has shown that in order to achieve an error of less than 10%, at least 700 cells need to be counted, given a specific scheme for selecting the quadrants in which the counting is performed. There are commercial solutions allowing to analyze microscopic images generating information e.g. about changes in the wood structure, also to recognize not only the number of cells but also to determine the number of dead and alive cells. Modern programming possibilities allow to create not complicated and low-budget application allowing to count single cells of specific strains of microorganisms, which has its wide practical justification.

Material and methods

Four strains of microorganisms in the collection of the Laboratory of Experimental Research Techniques of Biological Products and Raw Materials were selected for the study. Two bacteria (Enterococcus faecalis ATCC 29212 and Pseudomonas aeruginosa ATCC 27859) differing in shape and thickness of the cell wall and two yeasts (Candida albicans ATCC 90028 and Saccharomyces cerevisiae) differing in cell size were selected. In order to restore vital functions, reduction cultures were conducted on TSA solid medium (BioMaxima, Poland) in petri dishes (fig. 1 and 2).

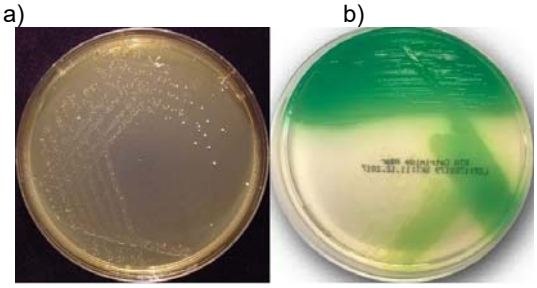

Fig. 1. Bacteria grown on petri dishes used in study: a) Enterococcus faecalis ATCC 29212; b) Pseudomonas aeruginosa ATCC 27859

Using a disposable ezy, colony fragments were taken and cultures were performed. Activities were carried out in a laminar flow cabinet under sterile conditions to prevent contamination. Plates were incubated for 24 hours at 37°C. After culture, the obtained colonies of individual microorganisms were suspended in sterile saline solution.

a) b)

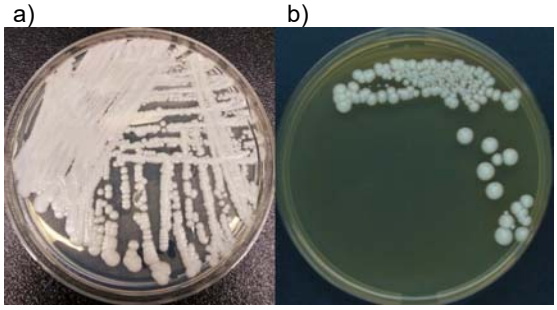

Fig. 2. Yeasts grown on petri dishes used in study: a) Candida albicans ATCC 90028; b) Saccharomyces cerevisiae

Four primary slides were prepared and degreased beforehand. 0.01 cm^3 of the tested cell suspensions of microorganisms were transferred to the slides, which were spread over an area of 4 cm^2 in each, making a smear. The slides were fixed by pulling the slide through the burner flame three times. After cooling, the slides were stained. Bacteria (*Enterococcus faeca*lis ATCC 29212, *Pseudomonas aeruginosa* ATCC 27859) were stained by Gram stain. *Candida albicans* ATCC 90028 was stained negatively with nigrosin, while *Saccharomyces cerevisiae* was stained with methylene blue. Microscopic images of microorganisms were used for quantitative identification. The resulting microscopic images are shown in figures 3 and 4: Candida albicans ATCC 90028 (Fig. 3a), Enterococcus faecalis ATCC 29212 (Fig. 3b), Pseudomonas aeruginosa ATCC 27859 (Fig. 4a) and Saccharomyces cerevisiae (Fig. 4b).

a) b)

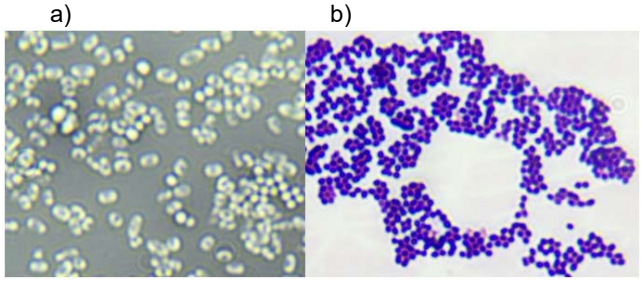

Fig. 3. Microscopic image: (a) *Candida albicans* ATCC 90028, (b) *Enterococcus faecalis* ATCC 29212

a) b)

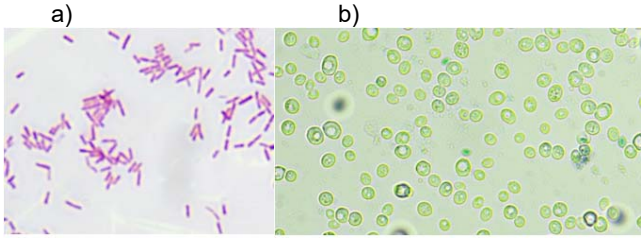

Fig. 4. Microscopic image: (a) *Pseudomonas aeruginosa* ATCC 27859, (b) *Saccharomyces cerevisiae*

The slides were viewed under a microscope. Microbial cells from 20 fields of view were counted in each slide. Cell counts were determined using the Breed method. The number of microorganisms was determined from the relation 1:

$$L = \frac{Ax}{60\pi r^2} * 100 \quad (1)$$

where: L - number of microorganisms present in 1 cm^3 of cell suspension; A - smear area; x - number of cells counted in 60 fields of view; πr^2 - area of the field of view

πr^2 = 0,36 cm^2 – for 400x magnification (*Candida albicans* ATCC 90028, *Saccharomyces cerevisiae*)
πr^2 = 0,16 cm^2 – for 1000x magnification (*Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27859)

The program makes use an OpenCV-Python project. It is a Python language library designed to help in computer vision problems. The project is distributed under the Open Source MIT License. Main method used to recognize the bacterial cells is "findContours" function. The function retrieves contours from the binary image using the algorithm described in [10]. Binarization of the image is made by “adaptiveThreshold” method, working according to the formulae:

$$dst(x,y) = \begin{cases} 0 & if src(x,y) > T(x,y) \\ maxValue & otherwise \end{cases} \quad (2)$$

Before binarization, image is preprocessed using “GaussianBlur” method, which helps to isolate the border of cells. Program uses also some additional functions from OpenCV library to read the image, to show and to write the final work. All functions used:

cv2.imread; cv2.imshow; cv2.imwrite; cv2.GaussianBlur; cv2.adaptiveThreshold; cv2.findContours; cv2.contourArea cv2.drawContours; cv2.putText

Validation of the identification of the number of microbial cells counted within the designated squares was performed based on the difference between the sum of cells counted in the traditional way and using the developed application. Using Dixon's test, the results were checked for coarse errors. The standard deviation of repeatability was calculated and the repeatability limit was determined from relation 3.

$$r = t_{(\alpha,f)} \cdot \sqrt{2} \cdot s_r \quad (3)$$

where: r - repeatability limit ; sr - standard deviation of repeatability; t(α,f) - value from Student's t-distribution for the number of degrees of freedom f = n-1 and significance level of 95% (in the study the value of 2 was assumed).

The reproducibility parameters, which were reproducibility standard deviation (sR), reproducibility limit (R) and reproducibility coefficient of variation (%sR), were then calculated. The variance describing the between-run variability was determined from relation 4.

$$s_{Lj}{}^2 = \frac{s_{dj}{}^2 - s_{rj}{}^2}{\overline{n_j}} \quad (4)$$

where:

$$s_{dj}{}^2 = \frac{1}{p-1} \cdot \sum_{i=1}^{p} n_{ij} \cdot \left(\overline{y_{ij}} - \overline{\overline{y_j}}\right)^2 \quad (5)$$

S_{lj}^2 – between-series variance; y_{lj} – average; y_j – overall average

However, the reproducibility limit from relation 6.

$$R = t_{(\alpha,f)} \cdot \sqrt{2} \cdot s_R \tag{6}$$

The final element of the validation was to determine the standard uncertainty (uc) and extended uncertainty (U).

Results

A computer program identified microbial cell counts in three steps. Each microscopic image was converted to a binary image and then cell shape identification was performed. After the shape identification, the application performed the process of counting the cells so identified by giving their total value from the area of the photo loaded into the computer. Figure 5 shows an example of the process of operation of the program on macroscopic images of microorganisms *Candida albicans* strain ATCC 90028.

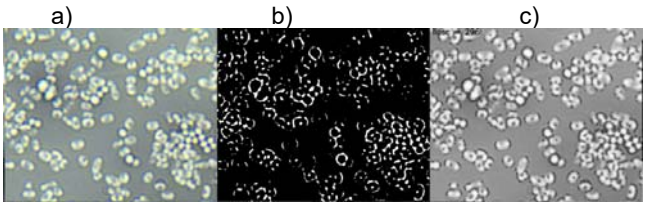

Fig. 5. Microscopic image of *Candida albicans* ATCC 90028: (a) microscopic image, (b) binarized microscopic image, (c) microscopic image after identification of yeast abundance

It was found that the mean value of yeast count determined by software for *Candida albicans* strain ATCC 90028 was 293.6 units. The repeatability deviation was 29.3 units and the repeatability limit was 82.9 units with a repeatability coefficient of variation of 9.98%. In the analyzed case, the reproducibility deviation was 27.0 units and the reproducibility limit was 76.5 units, while the reproducibility coefficient of variation was 9.21%. Thus, the expanded uncertainty in absolute values was 293.6±56.2 units and the expanded uncertainty as a percentage of the obtained result was 19.14%.

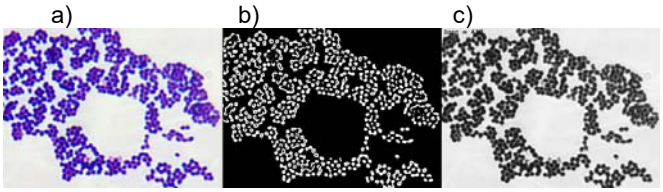

Fig. 6. Microscopic photo of *Enterococcus faecalis* ATCC 29212: (a) microscopic photo, (b) binned microscopic photo, (c) microscopic photo after identification of bacterial abundance

Figure 6 shows the cell counting process based on microscopic images of microorganisms. It should be noted that the shape and position of the cells is different in relation to the microorganisms *Enterococcus faecalis* ATCC 29212.

The analysis of *Enterococcus faecalis* ATCC 29212 cell counts determined by the program on the basis of microscopic images showed similar accuracy of identification of the counts of the cells in question in relation to *Candida albicans* ATCC 90028 strain [14]. In this case, the average bacterial count was 427.6 units. However, the repeatability deviation was 32.8 units and the repeatability limit was 92.9 units with a repeatability coefficient of variation of 7.68%. In the analyzed case, the reproducibility deviation was 35.6 units and the reproducibility limit was 100.7 units, while the reproducibility coefficient of variation was 8.33%. Thus, the expanded uncertainty in absolute values was 427.6±68.78 units and the expanded uncertainty as a percentage of the obtained result was 16.09%.

In the case of *Pseudomonas aeruginosa* strain ATCC 27859, it was observed that the shape of the bacteria was significantly different from that of the other microorganisms (Figure 7). In processing the microscopic image of the strain in question, a very accurate quantitative identification was observed by the developed application. The mean number of *Pseudomonas aeruginosa* ATCC 27859 bacteria in suspension was 134.9 units and the repeatability deviation was 4.5 units with a repeatability limit of 12.6 units and a repeatability coefficient of variation of 3.31%.

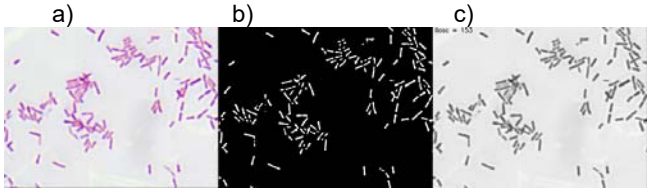

Fig. 7. Microscopic image of *Pseudomonas aeruginosa* ATCC 27859: (a) microscopic image, (b) binned microscopic image, (c) microscopic image after identification of bacterial abundance

In the case analyzed, the reproducibility deviation was 4.2 units and the reproducibility limit was 11.9 units, while the reproducibility coefficient of variation was 3.12%. Thus, the expanded uncertainty in absolute values was 134.9±8.67 units and the expanded uncertainty as a percentage of the obtained result was 4.43%.

Figure 8 shows the process of cell counting from microscopic images of *Saccharomyces cerevisiae* microorganisms in suspension. It was found that the average value of the number of automatically identified cells was 163.8 cells. The repeatability deviation was 9.1 units with a repeatability limit of 25.7 units and a repeatability coefficient of variation of 5.55%.

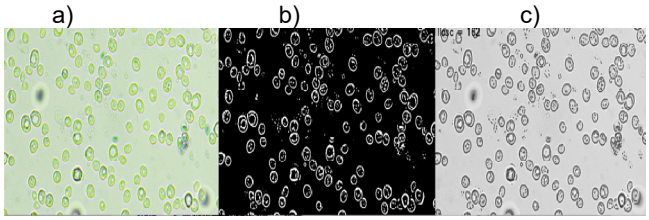

Fig. 8. Microscope photograph of *Saccharomyces cerevisiae*: (a) microscope photo, (b) binarized microscope photo, (c) microscope photo after identification of yeast abundance

When the reproducibility parameter of the measurement results was analyzed, it was found that the reproducibility deviation was 8.32 units and the reproducibility limit was 23.5 units, and the coefficient of variation of reproducibility was 5.07%. Thus, in summary, the expanded uncertainty in absolute values was 163.8±17.37 units and the expanded uncertainty as a percentage of the obtained result value was 10.6%.

Conclusion

The developed software allowed, in the case of the bacterium *Pseudomonas aeruginosa* ATCC 27859, to achieve an accuracy (expanded uncertainty of 4.43%) higher than traditional methods for bacterial counting. In the case of other microorganisms strains, satisfactory accuracy was achieved and comparable to traditional methods. It should be noted that the technology used to build the software allows for its modification to match the specificity of shape recognition and subsequent cell summation to a

particular strain of microorganisms, which will improve the accuracy of quantitative identification of microorganisms in biological suspension.

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