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SOFTWARE DEVELOPMENT FOR OBJECTIVE AUTOMATIC COUNTING OF LACTIC ACID BACTERIAL COLONIES GROWN IN MRS AGAR

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Abstract

Microbiological analyses are a significant part of food production and food quality evaluation. A commonly used method for food quality evaluation is the identification whether the food is contaminated with microbiological agents or not. This identification is performed by determining the total microbial number in a taken certain sample which (determining) can be done by counting bacterial colonies. Nowadays, there are manual, semi-automatic and automatic methods for counting bacterial colonies. The automatic methods require the usage of special systems rather quite expensive hardware and software. In comparison the manual counting is a cheaper solution but it is a time consuming method and it is subjective. This paper presents a computer based approach for objective and automatic counting of lactic acid bacterial colonies grown in a MRS agar.

The research is based on a previous research of possibilities for application of open-source software for microbiological analyses. A group of petri dishes with MRS agar and *Lactobacillus plantarum* Pro are captured by a digital camera. A software for digital images processing with graphical user interface is developed. The application is used for automatic detection and counting of bacterial colonies. It is developed in object oriented programing language Java as a plug-in for a well-known open-source software – ImageJ. The program is tested in the University of Food Technologies - Plovdiv.

A comparison analyze of the results obtained using the developed software is performed with the results obtained using manual counting.

The analysis shows that the proposed software can be successfully used for automatic detection and counting of the lactic acid bacterial colonies grown in the MRS agar.

***Key words**: Digital image processing (DIP), Bacterial colonies counting, ImageJ, Lactobacillus plantarum Pro.*

1. Introduction

Varieties of bacterial strains are used for the production of food and beverages [1, 2]. The group of lactic acid bacteria is widely used in fermentation processes in order to preserve the quality of food for longer time in comparison with to raw foods [3]. In recent years, many researchers have worked to develop foods with specific benefits for human health such as bread and confectionary products [4, 5], milk beverages [6], meat products [7, 8], etc. In order to evaluate the parameters of the developed products it is necessary to identify specific bacterial colonies. There are manual, semi-automatic and automatic methods for counting bacterial colonies. The manual counting is the oldest method for identification of bacterial colonies, but it is a time consuming and the accuracy of the results depends mainly on the experience of the performer. The semi-automatic counting is a popular approach which uses special hardware to support the accuracy of the performance. The hardware has a specific illumination and accessories (additional illumination, magnifiers) in order to improve conditions for the performer (human). The automatic counting is based on an images processing. There are a lot of apparatuses for automatic counting of bacterial colonies grown on specific medium. Most of these apparatuses are used for medical applications and their price is very high. Lately, many researchers have presented software tools

for automatic counting of specific bacterial colonies [9, 10 and 11]. A research on possibilities for application of open-source software in bacterial colonies counting, is performed using plates with *Lactobacillus plantarum* BOM2 [12].

The aim of this paper is to propose a software application for automatic colonies counting of lactic acid bacteria grown in MRS agar.

2. Materials and Methods

2.1 *Lactobacillus plantarum* Pro

A strain of lactic acid bacteria - *Lactobacillus plantarum* Pro is used for analyzing the efficiency of the proposed software. The strain Pro has proven probiotic characteristics and it can be used successfully for quality improvement of some probiotic functional drinks containing alive lactic acid bacteria [13, 14]. A bacterial starter culture of this strain is insulated and kept in the department of Biotechnology in the University of Food Technologies - Plovdiv.

For the purpose of this study eleven plastic agar plates with colonies formed by lactic acid bacteria - *Lactobacillus plantarum* Pro are analyzed. The colonies are grown in MRS agar. The bacteria form round shaped white colonies by their growing. Each of the agar plates is captured in a daylight environment (Figure 1 b) and in a laboratory setting with controlled artificial lighting (Figure 1 a).

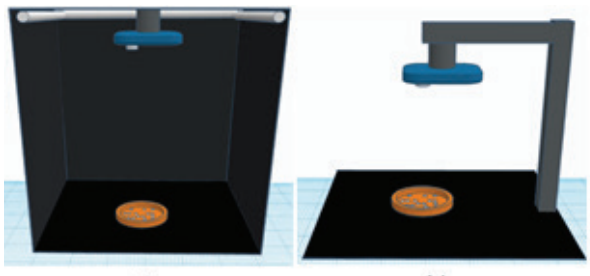

Figure 1. Experimental setting for artificial lightening

An 8MP digital camera integrated in a portable mobile device is used and the photos are stored as a bitmap images. A background unification technique is used. The black color background is chosen because it has good contrast with agar plates. The photos of all agar plates are shown on Figure 2.

The number of the colonies varies in each Petri dish (see Figure 2). The reason for this is the different stages of growth and the different concentration of lactic acid bacteria spread on the surface of the MRS agar.

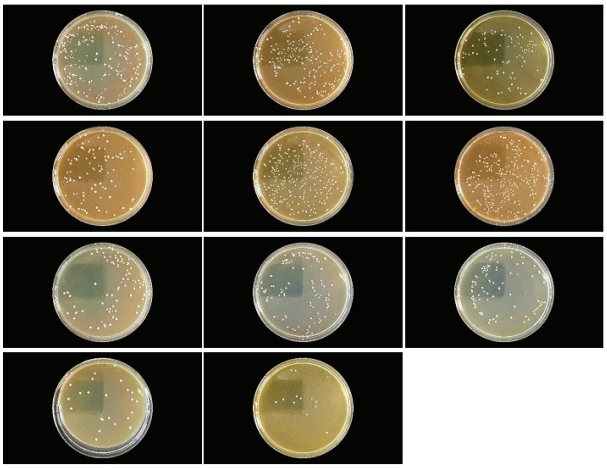

Figure 2. Agar plates images captured in a daylight

The difference in the color of the agar medium is a consequence of the difference of the agar thickness because the agar medium is manually poured in the plates while it is in a liquid state. The agar plates are plastic and their diameter is 12 cm.

2.2 Workflow of the program and performing an analysis

For the purpose of this research an open-source program called ImageJ is used as a backbone for developing a plugin called *ColonyCounterPlugin*. As a program with open license ImageJ provides huge amount of tools, functions, methods and libraries for developing unique software products in the field of image analyses [14]. A researches show that ImageJ is successfully used for developing software solutions in different areas such as microbiology, medicine and other [15, 16, 17 and 18].

Figure 3 shows a sequence of ten steps presenting the workflow of the program and the steps needed to be done in order to perform an analysis. In the beginning, the user has to open the program ImageJ (1) and to load an image to be analyzed (2). The image formats supported by the ImageJ are standard image formats such as: TIFF, GIF, JPEG, PNG, BMP, and others [19].

As a third and a fourth step, the user have to choose the *Plugin* menu from the ImageJ interface and start the program ColonyCounterPlugin using the submenu. After completing these initial steps a main window of the plugin opens. It is shown on Figure 4. As a next step, the user have to select the desired region of interest with pressing the button "Set ROI" from the user interface of the ColonyCounterPlugin (5) (Figure 4). The program is developed in such a way that the dimensions and the position of the ROI is implemented as a perfect circle which matches the inner diameter of the agar plate where the growing medium is poured.

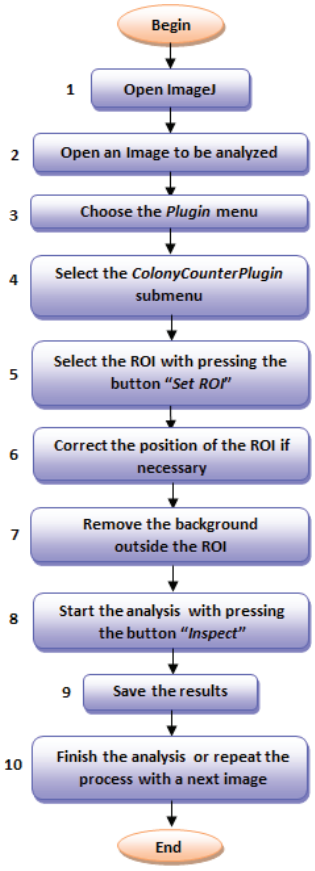

Figure 3. Workflow of the program

However, sometimes it is necessary to correct the dimensions and the position of the ROI. This operation can be done manually (6) (Figure 4). As a next step, the background outside of the ROI must be removed. It can be done by pressing the button "Clear outside" which calls a function that erase the background outside of the ROI by color conversion of all pixels from RGB to black. The process of automatic detection and counting of bacterial colonies starts with pressing the "Inspect" button (8) (Figure 4). In this step the plugin performs a sequence of image processing functions in order to detect and count all colonies. The processing includes:

- Smoothing the image.

- Subtracting the background representing the growing medium.

- Converting the image to gray scale.

- Using a threshold algorithm for image binarization.

- Using the Watershed function for separating overlapped colonies.

- Using Analyze Particles or Find maxima function.

The results of the analysis performed are shown on Figure 5. An image of all detected colonies is shown on the left side of the user interface of the program. Each colony is outlined and numbered. On the right side of the user interface in a table view format are presented useful information such as: the name of the current file; the number of all counted colonies; the total area of all colonies (in pixels); the average size of detected colonies; the area of the colonies in percentages and other.

All the results can be saved in .xlsx file format with pressing the button "Save Results". The results table can be cleared using the button "Clear results" if necessary. As a final step, next image can be loaded (the button Next Image) from the root directory containing all images for processing or an image can be loaded from another directory using the "Browse" button.

2.3 Plugin development for open-source software ImageJ

The plugin is developed in *Java* language using Eclipse IDE for Java Developers, version Neon.3 Release (4.6.3).

Figure 4. Main window of the plugin

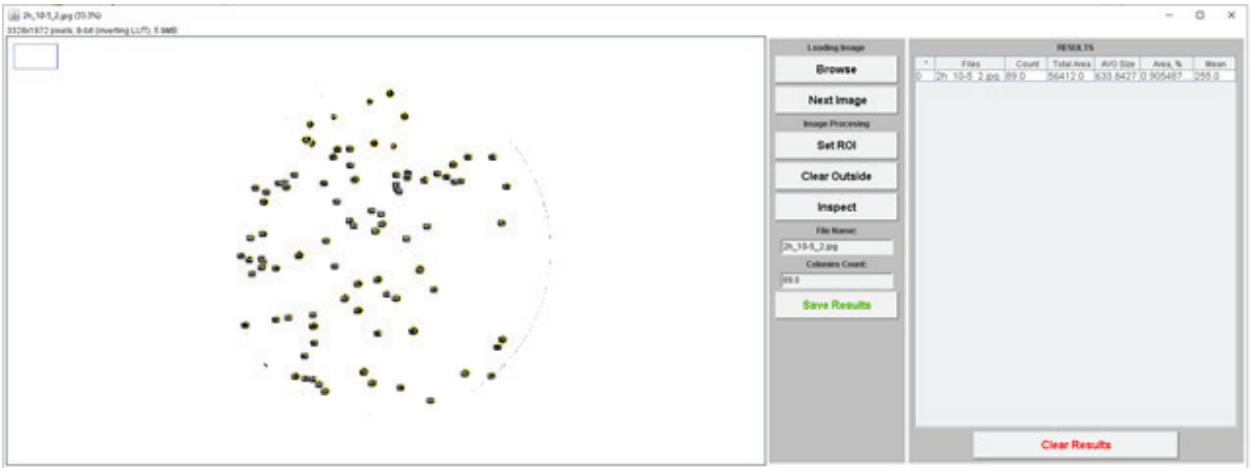

Figure 5. The results after image analysis

The program works in conjunction with the open-source ImageJ library and uses some of its built in classes and functions. The main project of the plugin consists of several directories. There is directory for each one of the source code files (src); a directory containing the images for analysis; one directory for saving the results (Results) and one directory for the files for different ROIs (ROI). The file hierarchy of the plugin is shown on Figure 6. The plugin consists of two main classes: ColonyCounterPluginV1_.java and Side_Panel.java. The first class defines the run*()* method which starts the plugin and sets some initial parameters such as dimensions of the main window and the position of the window on the PC monitor. The second class "Side Panel" which extends JPanel, defines all variables, objects and methods for creating the user interface of the program. The class "Side_Panel" contains a subclass called "CustomWindow" which extends ImageWindow. In "CustomWindow" class all objects representing fields, buttons and image canvas from the user interface are created and necessary parameters are set. A methods called actionPerformed*()* is used for performing all actions related with pressing the buttons, visualizing the results and other.

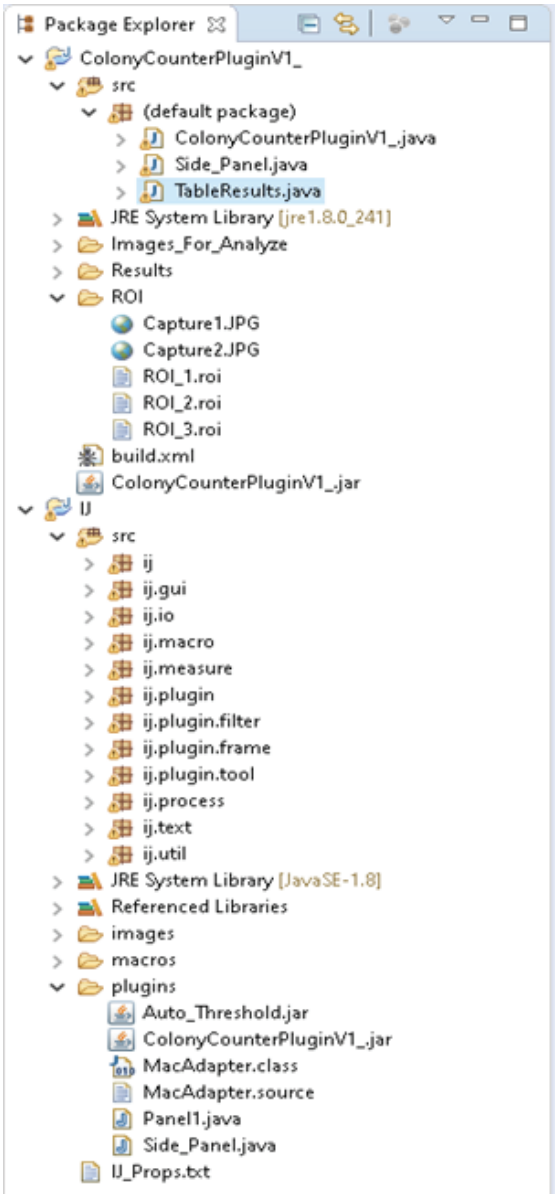

Figure 6. Files hierarchy of the plugin

3. Results and Discussion

In order to test the efficiency of the proposed software 11 images of agar plates (Figure 1) are analyzed. All the agar plates are captured using a digital camera with 8MP resolution and the images are taken by two different approaches - using daylight and using controlled artificial lighting. A comparative study between these approaches is performed and the results are shown graphically on Figure 7 a) and b). An additional comparative study is made related to the methods for automatic detection that are used. The results are presented in Figure 7.

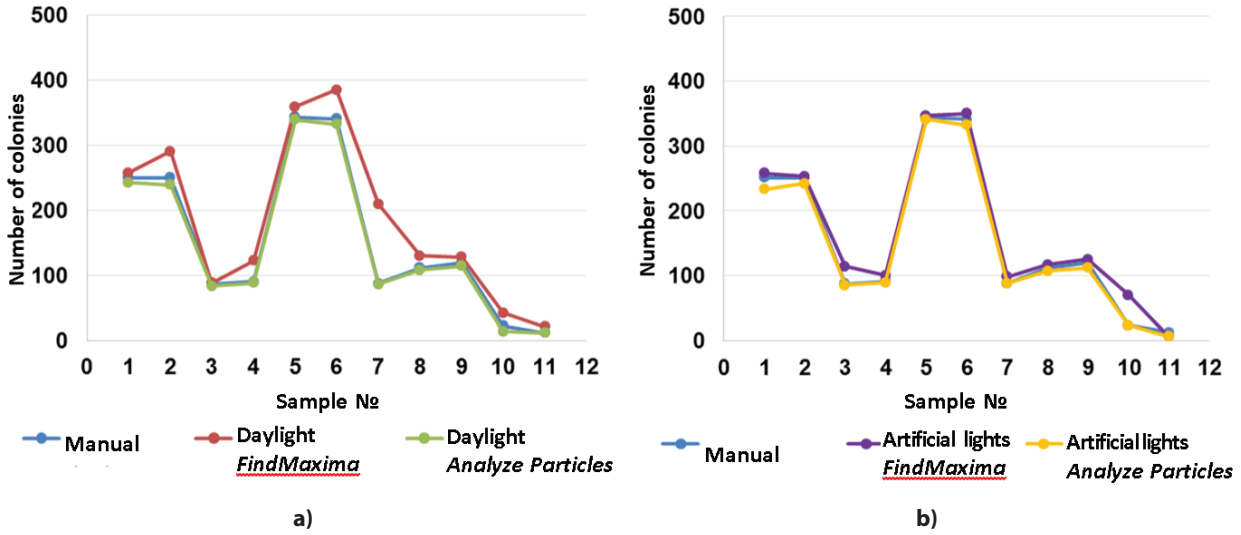

Figure 7. Comparative analysis between daylight and artificial lightening

A method for automatic detection and counting of objects called "Analyze Particles" is chosen because of its higher accuracy. Figure 7 a) presents graphics for the results obtained by manual counting and counting of the colonies by using the plugin and images captured in daylight environment and Figure 7 b) presents the results after capturing the images in laboratory settings with artificial lightening.

Each agar plate contains different number of colonies with varying sizes, different growing medium thickness (which leads to variations in the colors of the medium) and in some plates there are groups of colonies that overlap each other. These are some of the problems that have been addressed and solved in the program development process.

The results obtained after automatic detection of bacterial colonies of *Lactobacillus plantarum* Pro are shown on Table 1. As a reference method a manual counting of the colonies in all agar plates is performed. The table shows the amount of colonies counted using the developed plugin. A variation information for each analyzed agar plate is presented. An absolute error ΔA (in the number of the colonies) is calculated. After processing the data a relative error is calculated too. The relative error (in percentages) is 2.14 % as seen on Table 1.

Table 1. The results for colonies count using manual counting and using the developed plugin

***Lactobacillus Plantarum* Pro**			
Image	**Manual Counted Colonies**	**Using *ColonyCounterPlugin***	
		Counted colonies	**Variation**
Sample 1	251	236	15
Sample 2	251	240	11
Sample 3	87	86	1
Sample 4	91	89	2
Sample 5	344	340	4
Sample 6	341	336	5
Sample 7	88	85	3
Sample 8	112	110	2
Sample 9	120	120	0
Sample 10	23	30	7
Sample 11	12	12	0
Sum	**1720**	**1684**	**50**
ΔA		**36,00**	
Relative Error, [%]		**2.14**	

4. Conclusions

- A software for automatic counting of lactic acid bacterial colonies (*Lactobacillus plantarum* Pro) is developed. The program is implemented as a plugin in the open-source computer software for digital image processing ImageJ. The plugin is based on an algorithm which uses build-in functionalities and tools in ImageJ.

- Eleven agar plates with different number of colonies are captured by digital camera and they are analyzed in order to evaluate the accuracy of the developed plugin. The results after automatic detecting and counting the

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colonies are compared to the results obtained from manual counting. A percentage of relevant error (2.14 %) is calculated.

- Regarding the results it can be concluded that the developed software can be successfully used to determine the total number of bacterial colonies grown on MRS agar and the software can be used in the processes of microbiological and biotechnological analyses.

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