„Dunărea de Jos" University of Galaţi

Doctoral School of Fundamental and Engineering Sciences

PHD THESIS

- PhD Thesis Summary -

CONTRIBUTIONS ON THE USE OF IMAGE PROCESSING TECHNIQUES IN CONTROL AND ANALYSIS OF BIOPROCESSES

PhD student,

Eng. Laurenţiu Marius BAICU

Scientific supervisor,

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Series I8: System engineering No. 3

GALAŢI

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Introduction

Bioprocesses

Bioengineering is a science that involves biological processes, processes in which microorganisms cultures develop. These are called biotechnological processes or bioprocesses. Biosynthetic processes (biodegradation), such as those for the production of enzymes, medicines (antibiotics, vitamins etc.), organic acids, biofuels, pigments etc. can be listed as biotechnological processes. Biomass production processes (e.g. growing mushrooms) and, not less importantly, biological waste water treatment processes should be mentioned. Specific bioprocesses are that they involve the development of living organisms that produce naturally different products of interest to human communities. Obviously, the development of micro-organism cultures requires the provision of specific environmental conditions in which they grow and have high productivity and increased yield. Hence the necessity of approaching biotechnological processes in an interdisciplinary manner, understanding that, besides biotechnology, the growth of microorganisms in good conditions, other sciences such as automation (computer modeling and control of bioprocesses) contribute significantly (programming of computer systems for the implementation of automated algorithms), chemistry, physics, mathematics etc.

Generally, bioprocesses are carried out in specialized devices, called bioreactors. They are equipped with the equipment necessary for the development of cell cultures, including the automation elements (different transducers and specific execution elements). Structurally, a bioprocess involves two types of subsystems: the physicochemical subsystem and the molecular kinetics, which as a whole compete at the development of microorganism cultures. If the parameters of the physicochemical subsystems (pH, temperature, agitation speed etc.) it is not difficult to make their on-line measurement for the purpose of developing and implementing a driving algorithm (specific transducers at an acceptable cost price) in molecular kinetics subsystems, the problem of on-line information acquisition is much more difficult, meaning that the magnitude of interest (biomass concentrations, substrate, various metabolites, etc.) can not be measured on-line. The reason is related to the absence of sufficiently precise, reliable and not expensive cost-effective sensors that provide real-time information about these variables, information usable in control algorithms. Another possibility of data acquisition in the process is the use of state observers and parameters (state and parameter estimators). It is a frequent approach in control systems, generally good, but it is inconvenient to determine the mathematical model as accurate as possible to implement the estimator, and not to the ubiquity that biotechnological processes are non-linear, strongly affected by parametric or model uncertainties (hidden dynamics) and measurement and process noise. It therefore points out that a very important issue in the good performance analysis and control of a biotechnological process is the measurement of the variables of interest on-line.

In the research carried out within the present PhD thesis two main objectives were pursued, concretized in two research directions: the first objective is the realization of a system of measuring the variables of interest in bioprocesses in order to control it. Both the parameters of the physicochemical subsystem and, in particular, those in the molecular kinetics subsystem are concerned. In other words, it is desired to close the control loops associated with a bioprocess run in a bioreactor by means of measurement systems based on image processing techniques. A case study has been chosen: the growth of yeast cells of the species *Sacharomices cerevisiae*. The idea developed in the paper is to determine the concentration of cells of this species (biomass concentration) to provide the measured variable necessary for the operation of a bioprocess control algorithm by image processing techniques.

The second direction of the research was the use of image processing techniques in the diagnosis of biotechnological processes. For this purpose, images were captured at certain time intervals using a microscope camera by a human operator, images that were then used in image processing algorithms for cell counting and then classifying them into

classes, such as: young cells, cells in division (capable of multiplying), mature cells, old cells, dead cells.

The researches in this paper have an interdisciplinary character, being carried out within the Bioaliment research platform of "Dunarea de Jos" University of Galati, in collaboration with the staff of the platform laboratories.

The thesis is structured as follows: an introduction, four chapters, conclusions and original contributions of the PhD thesis as well as two annexes.

The introduction presents the motivation for choosing the research theme approached in the PhD thesis and its structure.

Chapter 1 presents the current state of research on image processing applications in bioprocess management. This chapter includes the methods currently used in microscopy, image processing, and systems used in cell culture research. Also, there are a number of types of status observers and parameters used to measure the magnitude of interest in bioprocesses.

Chapter 2 presents contributions to the application of image processing techniques in bioprocess control. An experimental stand consisting of a bioreactor and the associated equipment (heating jacket, agitator, temperature sensors, pH, turbidity), auxiliary systems such as: peristaltic pumps, bypass to take samples from culture, microscope, turbidity controller , the computerized measurement and control system consisting of a computer equipped with an acquisition board, a connection interface between the acquisition board and the bioreactor subassemblies, and, last but not least, the color camera for real-time acquisition of images. In order to link the bioreactor subsystems to the computer, it was necessary to provide a specific interface that would allow the acquisition of the input signals from the sensors, transmit them to the acquisition plate, and receive the output signals from the acquisition board and to send them to the execution elements

Capturing in-situ images of real-time cell culture required the design and realization of a new element specific to the biomass concentration sensor (flow and observation cell, also called bypass). For this purpose, more research and tests have been carried out to determine the best construction option to meet all the necessary requirements. Finally, a method for determining the biomass concentration, which can be used in an automated control algorithm, has been developed. Thus, an original method for determining the biomass concentration was developed by calculating the individual cell volume individually, summing the cell volumes and reporting to the observation volume (image size, bypass flow thickness).

In Chapter 3, the algorithm for biomass concentration determination has been validated by image processing techniques. In order to control the biomass concentration so that the system can operate fully automated, it was necessary to develop original software in Matlab environment that can retrieve the input data from the acquisition board, process them and determine real-time controls applied to the bioreactor's actuators. The optimal parameters of this software were determined on the basis of several tests and experiments. Practically, the algorithm for determining biomass concentration by image processing techniques has been applied to a yeast cell bioprocess of *Sacharomices cerevisiae* species. The algorithm for determining biomass concentration by image processing techniques was included in a biomass concentration control loop.

Chapter 4 refers to the diagnosis of bioprocesses (in the sense of being able to appreciate the state of cell culture development) using image processing techniques. The diagnosis consists of analyzing the evolution of bioprocesses based on the physiological state of the cell culture. For this purpose, a new concept was introduced to characterize the physiological state of microorganisms, the degree of cell viability, which was determined using black and white images without using classical invasive techniques (staining with methylene blue, etc.). An original contribution is to develop an algorithm capable of determining the viability of the cell (live, dead), cell count, and biomass concentration of

more than 80% accuracy based on their brightness analysis. Validation was performed using a Phase-contrast Olympus BX41 microscope.

Also, the development stage and cell classification were evaluated based on the information obtained from color image analysis. An experiment was conducted to determine the different stages of culture development on the yeast growth process of *Saccharomyces cerevisiae* on a standard culture medium MEA (Malt Agar Extract) from the Microorganism Collection of the BIOALIMENT Research Platform of the "Dunarea de Jos" University of Galati.

Two experimental versions were carried out: one on Yeast Extract Glucose medium with an optimal nutrient content required for yeast growth and multiplication and the other on an auxotrophic G medium without nitrogen source and therefore poor in nutrients . Following this experiment, the five cell states mentioned above were identified. Parameters determined by the inner texture of the cells were analyzed and non-viable / non-productive cells found inside dark areas (called ergastic inclusions). The experiment had a duration of five days.

An original algorithm has been developed to classify cells into five categories: young, mature, old, dead, using imaging techniques such as *k*-means clustering technique for identifying nucleotides within each cell. Validation was performed by several laboratory tests, such as optical density measurement (DO600) using the Hach Lange DR3900 Spectrophotometer, cell count in Standard Plate Count (SPC) cells, measurement of nitrogen, glucose concentration, etc. This algorithm can be adapted to other types of cells similar to geometry.

The last chapter is dedicated to the conclusions, original contributions and future research directions. The two annexes complement the PhD thesis with a number of elements regarding the equipment used in the experimental research within the thesis and the programs designed and implemented within it.

Chapter 1

The current state of the imaging process in the monitoring, management of the process and the processes

1.1 General biotechnological processes

Biotechnological processes are processes in which microorganisms (cell populations) are cultivated in order to obtain various biologically products. They have evolved as methods of obtaining various food products, pharmaceutical products and compounds, pigments, enzymes, biofuels, biopolymers etc., but also for the regeneration and protection of the environment, using live cells for this purpose. These are waste water treatment processes (aerobic or anaerobic) using so-called active sludges for the treatment of residues in water for the purpose of discharging them into natural environments. Bioprocesses bring a number of advantages due to the relatively high rate of multiplication of microorganisms, the advantages being of an industrial scale and, not the least, by the fact that the products are obtained by natural way.

A biotechnological process involves a set of chemical and biochemical reactions that take place in specific equipment called bioreactors. The bioreactor is an equipment that must provide conditions conducive to the development of the culture of microorganisms, that is to say, it must guarantee a non-limiting transfer of nutrients from the culture medium to the cells, regardless of how these devices have been designed. It has thus developed a science, called Bioprocessing Engineering, which specially deals with the study and operation of bioprocesses. Afterwards, there was a need to find ways to increase the efficiency of biotechnological processes, and in this sense, specialists in automation, computer science, mathematics, the field became interdisciplinary. It can be argued that mathematical modeling and automatic control methods have revolutionized the field of biotechnology, being further developed by mixed teams of specialists. In fact, the field of biotechnology has become a real challenge for automation specialists, with the challenges of designing and applying more and more evolved algorithms for bioprocesses, characterized by nonlinearities, uncertainties and process and measurement noises.

There are several criteria for the classification of biotechnological processes in the literature, two of which are:

- After the presence or absence of oxygen in the culture medium [1], [2]:
  - Aerobic processes - where the microorganisms consume oxygen, which facilitates the transfer of electrons;
  - Optional anaerobic processes - in which electron transfer can be accomplished through both oxygen and intermolecular transformations;
  - Strictly anaerobic processes - where oxygen is absent from the culture medium, and the transfer of electrons is accomplished through inorganic compounds.
- By bioprocessing mode:
  - Discontinuous processes (batch type or lot);
  - Semicontinuous (or fed-batch) processes - the process begins in batch mode and then switches to continuous feed mode;
  - Continuous processes.

In this paper the process of growth of S. Cerevisae yeast was approached in batch mode and then in semicontinuous and continuous modes. At the beginning, the process was developed in a batch mode by introducing a yeast culture into a sterile environment, containing all the necessary nutrients, and its evolution is monitored until the cell population

decline. The batch mode is characterized by several intermediate development phases as follows:

a. the lag phase: where the adaptation of the cell culture to the environmental conditions takes place, the population growth being practically zero;
b. intermediate phase (start of growth): where the size of the cells increases and their multiplication begins;
c. the exponential multiplication phase in which the number of cellules increases in geometric progression, so that the population doubles at regular intervals. This interval is called doubling time of biomass;
d. slowing phase of multiplication in which, as the nutrients deplete and the inhibitor compounds are formed, the rate of propagation decreases;
e. the stationary phase in which a maximum of cell culture is reached;
f. the decay phase, in which cell death causes population decline until extinction.

Figure 1.1 shows the sequence of the microorganism culture phases in batch development mode. It should be noted that these phases are found in other types of bioprocesses.

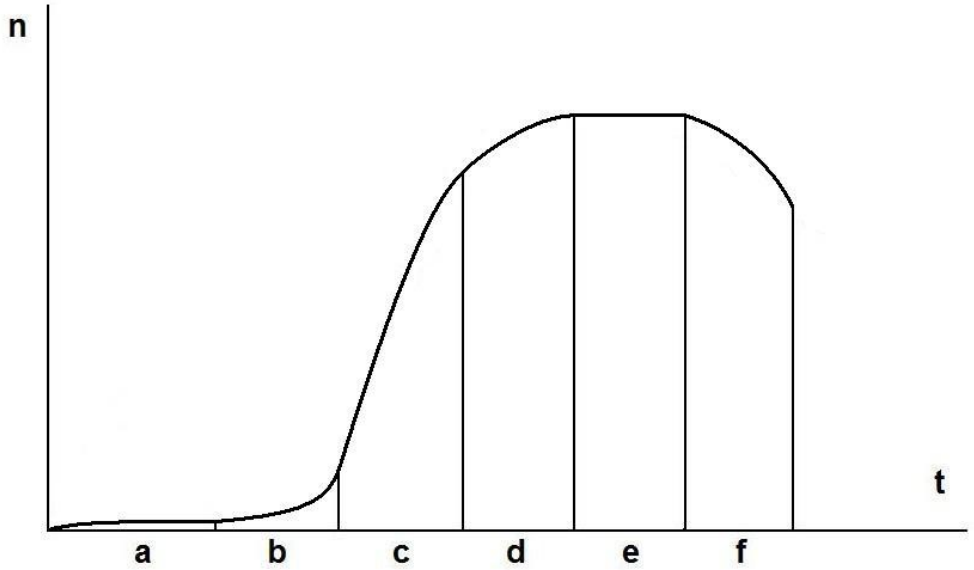


Fig.1.1 - Representation of the cell number according to the typical phases of culture evolution in discontinuous systems

Semicontinuous processes were performed by introducing a yeast culture into a bioreactor in a nutrient medium. During the evolution, the automatic system maintains the nutrient concentration to the preset value by means of an external pumping system as well as the pH and oxygen and nitrogen concentration at preset values. Continuous processes can maintain cell culture at a certain stage of development for an indefinite time as long as the yeast culture is fed with nutrient medium and at the same time eliminates an equivalent amount of used environment. At the same time, environmental conditions (temperature, pH, oxygen concentration, nitrogen, etc.) are constantly maintained.

1.2 Problems on the measurement of the values of the bioprocesses interest rates

Bioprocesses involve the development of cell cultures for production purposes. It is well known that cellular metabolism, which depends on their intracellular state and interactions with the environment, directly influences the productivity of bioprocesses. The operation of these processes is aimed at high productivity, and the efficient use of materials and energy resources is very important, which makes automation techniques (modeling and control) very useful and, at the same time, very necessary.

As mentioned, due to the complexity of these processes, the strong nonlinearities, the parametric or model uncertainties affecting these processes as well as the measurement or process noise accompanying the variables of interest, the modeling and control problems represent a real challenge for specialists. It is to be remembered that a slight change in cell culture conditions or crop contamination may cause changes in metabolism and hence a failure of the bioprocess with unintended consequences in the loss of important energy and material resources [3], [4].

An important problem of bioprocesses is the difficulty of measuring on-line the variables of interest due to the lack of dedicated sensors, reliable enough and at an acceptable cost price, which often makes it impossible to apply control algorithms to increase the bioprocess efficiency [5]. The use of software sensors (state and parameters, for example, measuring substrate and biomass concentrations, specific biomass growth rate, etc.) is a realistic solution in this case, and they are frequently used in control applications of bioprocesses [6]. The main difficulty that may arise in this case is the lack of a sufficiently precise mathematical model of the biotechnological process.

Another problem that can be mentioned in bioprocesses is the diagnosis of the process in order to determine the productive potential of cell culture. It is very important for the operator to appreciate at every moment the physiological state of the culture and how it will evolve in terms of productivity and efficiency. For example, information on culture contamination (eventually early detection of contamination) and the physiological state of the culture (if the culture has a high potential for growth or regress) would be extremely useful.

In the case of bioprocesses, on-line analysis of cells and obtaining data about the biomass kinetics results in better understanding and, obviously, better operating of the bioprocess. In collaboration, the conventional biomass growth rate is assessed by determining the dry mass in culture or by determining cell density, being off-line, time and effort-saving methods, requiring sampling, filtration or centrifugation, drying and weighing up to constant mass. While the determination of dry mass is relatively simple, cell density testing requires a counting chamber microscope and a trained eye to identify the cells in the field of view of the microscope. These determinations cannot be made on-line, limiting the possibility of automating the process. Alternatives to these methods can be the optical density determination (using a mass spectrophotometer) and the determination of the turbidity. However, determining cell density is the only method that can differentiate between cell and non-biological material, while other methods are not able to discriminate between them [2].

Chapter 2

Contributions for automatic measurement of biomass concentration in a bioprocess

2.1 Introduction

Use of biotechnological processes for the production of food, pigments, enzymes, pharmaceuticals etc. is a well-known practice, the engineering of this category of processes involves several sets of knowledge from the biological, technological (chemical, electrical, material science, hydraulics and electronics) as well as automation and computer science.

The purpose of this chapter is to present a method of measuring the biomass concentration, which reduces measuring time, reduces foreign-induced uncertainty (crop contamination) and delivers the result without operator intervention, thus eliminating its subjectivity. The validation of the method and of the concentration calculation algorithm was performed by comparison with classical methods.

For the measurement of biomass concentration, the current classical methods are grouped into:

- Counting cells in the Thoma room;
- Determination of dry mass (weighing);
- Use of turbidity sensors;
- Measurement of optical density.

The first two categories require the intervention of an operator and are strongly affected by his subjectivism. Moreover, the duration of the measurement is high, sometimes comparable to the time constants of the driven process, so it is costly and inappropriate for automatic driving. The last two categories can be applied online, so they reduce human influence and are faster, so they are more suited to an automated process.

Unfortunately, they cannot distinguish between bioreactor population cells and foreign bodies such as other invading cells, organic or inorganic impurities, materials deposited on the bioreactor wall, substances introduced during the process automatically or by the operator. As a result, their degree of certainty depends on the process being conducted and can be altered without the operator being warned. The classical methods are presented in more detail, since one of them will be used for the validation of the measurement algorithm.

From the above, one can conclude that a measurement method that can be applied on-line with a reasonable price is needed, but which can make the difference between the cells of the culture of interest in the bioreactor and other cells (possibly impurities). The method proposed in this chapter is based on the analysis of cell images, their recognition and counting being similar to the way a human operator would do. Subsequently, based on this method, a biomass concentration transducer, suitable for the management of biotechnological processes (Chapter 3) will be designed and constructed. Examining the images taken from the microscope has the advantage of allowing the analysis of the biological state of the cells and the detection of foreign elements, as can be seen in Chapter 4.

2.3 General principles of automated biomass concentration measurement

As anticipated, the proposed method consists of:

- automatically extracting a sample from the bioreactor content and exposing the liquid suspension to the microscope;
- acquisition of the image in the form of a formatted variable;
- analyzing the image by detecting cell populations, separating cell images from other objects, and measuring their geometric dimensions;

- calculation of the concentration based on geometric dimensions and transmission of its value to other subprograms.

The essential part of the proposed measurement method is to analyze the image, similar to the way a human operator would do, with the mention that its subjectivity is avoided. In the image analysis, the following steps take place:

- capturing an image of the sample;
- bringing the parameters of this image to values that give the best results after processing;
- separating the sub-images of the monitored population cells from the image
- separating the sub-images of the monitored population cells from the image captured by the camera, neglecting (at the same time) the objects of no interest (impurities, etc.);
- measurement of cell areas by automatic image analysis; - calculating cell volumes (this depends on cell shape);
- the biomass concentration calculation, using the cumulative volume of the cells, the volume in which the image (observed volume) was taken and the known cell density.

2.4 Algorithm for the measurement of biomass concentration by image processing

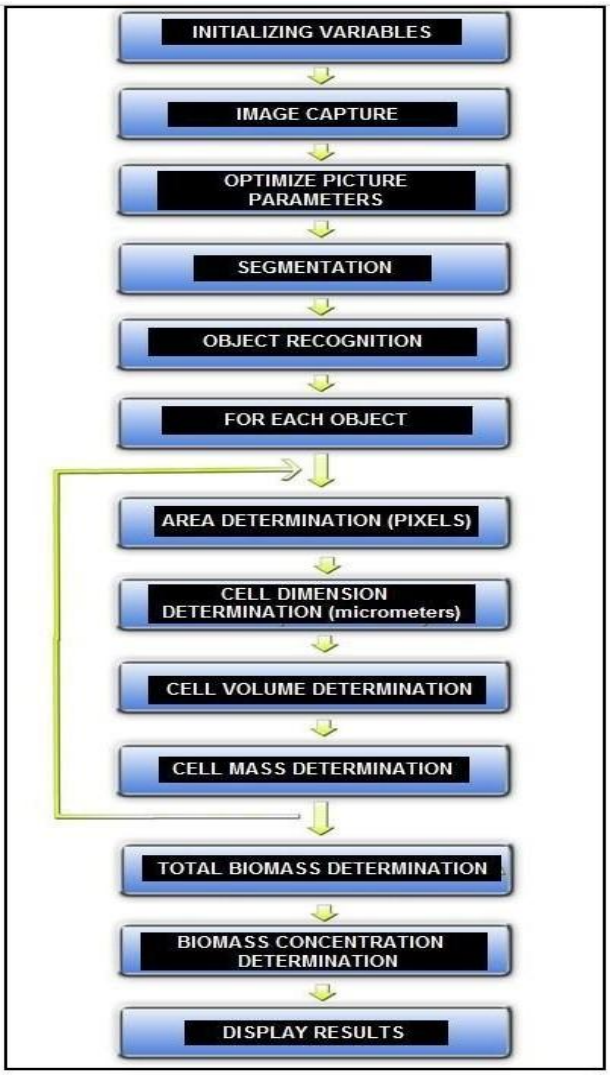
To apply the concentration measurement method presented in the previous subchapter, an algorithm for image processing is required. In the following, the algorithm developed for the aforementioned cell population class is presented. This algorithm was published in the paper [72]. The exemplification of the algorithm is also applied to the species Saccharomyces cerevisiae, which is included in this class of populations. It is assumed that a microscope image has already been acquired and that the resolution provided by the camera is reasonable (areas of the order of one thousand pixels per cell). As presented in the measurement method, it is a matter of separating cell images from other impurities, measuring their areas, then determining cell volume and concentration. The algorithm diagram is shown in Fig. 2.9.

In the first step, after the acquisition of the image, there is a change in global image parameters (brightness, contrast) so that the values of these parameters are as close as possible to the whole set of images. This change is required because the overall image characteristics may vary a lot, depending on content, focus etc., so that the processing results become inaccurate and negatively affect cell detection.

In essence, the following scale transformations take place:

- the luminance is changed so that the overall luminance average is close to a required value;
- overall contrast is brought to a value that allows cell differentiation (the luminous parts of the cell approach white, the membrane and the dark organ of the cell approach black, and the environment has an intermediate luminance).

Processing operations differ between the monochrome and the color variations. In the case of monochrome images, a specific method for eliminating the noise in the image, as well as unwanted reflections and impurities has been designed. In the case of color images, a different method was designed in view of the problems that arise in these images: a pronounced variation in brightness from the center to the periphery of the images and the focusing of the contours of the cells. These methods represent an important personal contribution and are detailed in Chapter 4.



Step 1: Initialize variables
Step 2: Acquire the image
Step 3: Determining the image parameters (contrast, luminance)
Step 4: Segment the image
Step 5: Recognize objects, tag and display detected cells
Step 6: For each detected cell, steps 7-10 will be applied
Step 7: Determine area in pixels
Step 8: Determine cell size (in micrometers)
Step 9: Determine cell volume
Step 10: Determine cell mass
Step 11: Determination of total biomass
Step 12: Determination of concentration
Step 13 : Display the results

Fig. 2.9 - The algorithm diagram for the measurement of biomass concentration

2.5 Implementation of the biomass concentration measurement algorithm

The part of the algorithm running on the computer can be programmed in any high-level language, but it has been preferred to use the Matlab environment, which has the entire set of operators for working with scalars and matrices, plus functions already implemented.

2.6 Validation of the biomass concentration measurement algorithm

Figures 2.16 - 2.17 and Table 2.2 show an example of calculating the biomass concentration using the algorithm described above. Fig. 2.16 is the image captured by the 1.3 mpx color camera. Fig. 2.17 is the processed image of the algorithm in which cells are identified and numbered. Table 2.2 shows the amounts of interest calculated by the algorithm for each cell, as well as the total wet, dry biomass concentration.

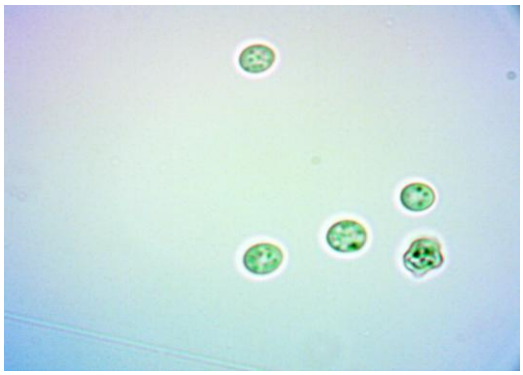


Fig. 2.16 The captured original image

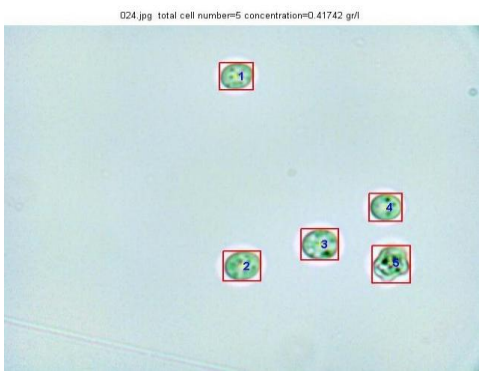


Fig. 2.17 Processed image with cell identification

Table 2.2 Dimensions of interest calculated using the algorithm

Cell number	1	2	3	4	5
Array [pixeli]	4951	6321	5996	4666	6875
Array [micron^2]	17.824	22.756	21.586	16.798	24.750
Diameter [microns]	4.764	5.383	5.242	4.6246	5.614
Volume [micron^3]	56.605	81.657	75.441	51.789	92.625
Total volume total of cells [cube microns]			358.1176		
Biomass concentration [gr/l]			0.4174		
Dry biomass concentration [gr/l]			0.0379		

Chapter 3

Contributions regarding realization of an automatic system for controlling the concentration of biomass using image processing techniques

3.1 Introduction

The application of these techniques has been done to determine the biomass concentration, but with a number of modifications of the algorithms projected in this work it can be applied to determine other variables, such as substrate concentrations, concentrations of metabolites etc. From the category of on-line measurement methods it can be mentioned:

- Determination of dry matter, which is a time and effort-consuming operation, this method requiring sample acquisition, filtration or centrifugation, drying and weighing up to a constant mass;
- Cell density determination, which requires a microscope equipped with a counting camera and a well-trained eye (by a specialist) to identify the cells in the field of view of the microscope.

Basically, a technique based on a conventional light field microscope and image processing methods for determining the biomass concentration and other parameters in biotechnological processes have been developed. This technique serves to implement a real-time automated system that can be used to automatically adjust some bioprocess interest rates.

3.2 The biomass concentration control system using image processing techniques

The two subsystems that make up the yeast development system were highlighted:

1. the microorganism kinetics subsystem and
2. the subsystem related to the physico-chemical parameters of the yeast culture.

Based on this decomposition, the control system contains three control loops as follows: the main loop is for the biomass concentration control, 2 secondary loops, the first for temperature control, and the second for the pH of the yeast culture control. The direct turbine command was considered for stirring the culture. The three control loops are considered independent, the temperature and pH loops having the role of providing a suitable operating point (a medium one) for the growth of the yeast culture of the species *Saccharomyces cerevisiae*. Fig. 3.2 shows the adjustment system in which a transducer based on image processing techniques taken from a microscope was used by means of a photodigital color microscope camera and processed with an average-performance PC equipped with an acquisition system.

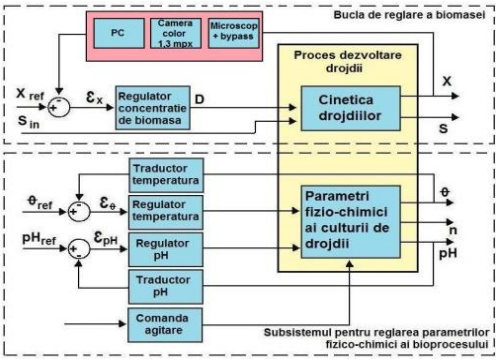


Fig. 3.2 – Biomass concentration control system. diagram

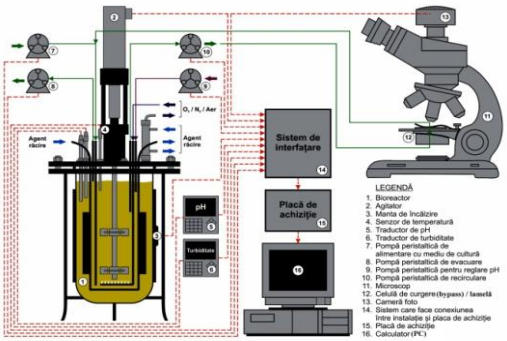


Fig. 3.3 - Controlled bioreactor

3.2.2. Sensor for measuring biomass concentration based on image processing techniques

The biomass concentration sensor consists of the following elements: a 60x lens microscope, a 1.3MPx photo/digital color camera, a bypass system (a flow and observation cell) and a peristaltic pump which recirculates the culture medium through the flow and observation cell.

1. **Microscope**: It is intended to facilitate the acquisition of images of the suspension in the culture medium with an appropriate degree of magnification in order to identify the cells individually. The microscope used is a standard one with two eyepieces and illumination using a halogen bulb. The objective used was 60x.
2. **Digital Photo Camera**: A 1.3 megapixel color digital camera has been used. The camera takes pictures with a period of about 10 minutes (see section 3.5). The magnification factor of the camera is equivalent to that of a 10x eyepiece and, therefore, taking into account the 60x lens of the microscope, it results that the images were captured with a magnification factor of 600x.
3. **Observation cell**: allows periodic recirculation of the suspension in the bioreactor vessel in view of the acquisition of images. This is detailed in section 3.3).
4. **Peristaltic pump**: is intended for recirculating the culture medium through the observation cell. It is a pump type SR25¬S300, presented in section 3.2.1.

3.3 Flow and observation cell

The flow cell is specially designed to be able to acquire real-time images taken from the culture medium, images of good quality for accurate image interpretation. The flow and observation cell, together with the photo-digital color camera, provide information to the computing system running the algorithm that analyzes images from the microscope and which ultimately determines the value of the biomass concentration. On this computational system is also implemented the biomass concentration control loop. The control variable provided by the controller is the dilution rate, which depends on the flow rate of the substrate metering pump to feed the bioreactor.

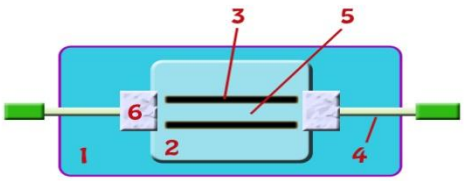


Fig. 3.4 - Bypass principle: 1 - lower blade, 2 - upper blade, 3 - flow channel partition walls, 4 - inlet / outlet tubes, 5 - flow channel, 6 - flowing - tubes

The entire flow path is completely sealed to eliminate the possibility of contamination of the culture medium. The flow and observation chamber is placed on the microscope table and can be fixed similarly to a normal microscope slide. Instead of one eye, the color camera is mounted and the flexible tubes necessary for the suspension recirculation path are connected.

In the biomass concentration adjustment algorithm, the computer controls the pump to re-circulate the slurry in the bioreactor vessel at a predetermined time interval, then capture images using the color camera mounted on a microscope, analyze them, and, depending by the evolution of culture, will generate the commands necessary to control the biomass concentration.

3.4 Implementation of real time software for the bioreactor control system

This was done in Matlab environment (RealTimeControl Toolbox - RTC) and uses drivers for communication with the Advantech interface board. The entire software structure works with a 1 second sampling period, with a graphical window viewing the evolution of the main parameters, as well as automatically saving as a file all the input and output parameters for further processing.

If the shutdown command is typed, the whole data acquisition and processing process stops and resets all the output commands of the bioreactor execution elements so that it enters a queuing state. Basically, there is no maximum limit value for the duration of the experiment, which is limited only by the capacity of the substrate reservoir feeding the feed bioreactor.

3.5 Transducer dynamics analysis

To evaluate delay introduced by transducer, the following experimental determinations were made:

1. the time required for the cell suspension to flow from the bioreactor into the flow cell (experimentally determined using a dye agent);
2. cell settling time in bypass to image capture (experimentally determined using successive images);
3. the time to calculate the biomass concentration.

A preliminary operation required to analyze the dynamics of the biomass concentration transducer using image processing techniques was to determine the real characteristic of the peristaltic recirculation of the culture medium in the bioreactor and calibrate it.

1. Determine the time required for the cell suspension to reach the bioreactor in the flow cell at the automatic pump start.

Experiment no. 1:

Initially, the peristaltic pump was started, which recirculated the bypass suspension using water, the pump was stopped, and a food color was poured into the water container to measure the amount of time the dye reached from the container in the bypass (Figure 3.10) being approximately 1.5 minutes for a 2V pump control voltage, corresponding to a flow rate of 7ml / min, and a coating value of about 2 minutes was chosen.

2. Determination of cell sedimentation time in bypass to capture images

Experiment no. 2:

The peristaltic biomass recirculation pump bypass was activated for two minutes, after which images were taken at intervals of 25 sec. After analyzing these images, it was found that biomass sedimentation ended after about 7 minutes. In the last image taken through real-time tracking of the flow of the suspension, the cells are in focus field stop moving and remain stationary.

3. Determination of the biomass concentration calculation time

This time depends directly on the computer system used in the application management process and can say, even in the case of a medium power, that time is insignificant compared to the other two delays set out in paragraphs 1 and 2. Basically, 2 minutes were allocated for "washing" the bypass circuit and the penetration into it of new cells from the container bioreactor and 8 minutes to stop the movement of cells and disposal thereof in all resulting in a sampling period of 10 minutes to sampling in order to capture images.

3.6 Validation of use of the biomass concentration measurement transducer based on imaging techniques in the yeast growth system

This was done in a control system, where the "classic" transducer (in the case of bioprocesses it can be a turbidimeter) was replaced by the bypass system presented in the previous section, together with the image processing algorithm. Particular emphasis was placed on the performance obtained by replacing a classical transducer or an observer with with a controller based on image processing techniques, with the advantages and disadvantages of using it in an automatic adjustment loop (ie on **the principle of measurement included in the adjustment loop**), and not on the control method itself. The design of the PI controller was experimentally performed. **The purpose of the design was not the realization of the best possible controller but of a sufficiently efficient one for a control system in order to validate the control method using the proposed transducer in this doctoral work, based on image processing techniques**. In the first phase, we identified the yeast growth process based on the process response.

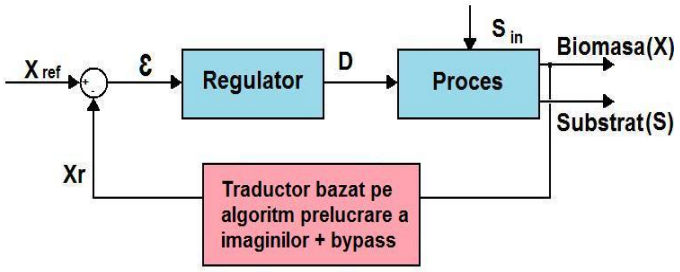


Fig. 3.33 - The block diagram of the biomass concentration control loop

Experiment no. 3:

The experiment was carried out continuously. The bioreactor was inoculated with 0.074 g / l of *S. cerevisiae* yeast. The bioreactor was fed with a substrate flow rate of 1 ml / min, which corresponds to a supply voltage of the substrate pump 1V, the substrate having a concentration of 25 g / l (sugar, nitrogen and potassium) at a constant temperature of 27- 28 ° C for about 4 hours. At the time t=250 minutes, a step was applied to the feed voltage of 0.4V substrate (from 1V to 1.4V), which corresponds to a variation in the flow rate from 1ml / minute to 5ml / minute (Figure 3.34 - the graph drawn in green), with the use of a 4 x 3.5 mm tube.

Table 3.2 shows the numerical values of the biomass concentration and the supply voltage of the substrate pump with a period of 10 minutes, as shown in section 3.5 of this chapter. The images were captured using the 1.3 mpx color digital camera, mounted on a microscope. . These were processed with the yeast cell identification algorithm and determination of biomass concentration presented in section 2.3 - Chapter 2.

In the following table, the two stationary regimes are marked in yellow: the first between 150 and 250 minutes, after which the peristaltic substation feed pump voltage step was applied and the second stationary mode obtained after applying the voltage step, between moments 550 and 730 minutes. From the table we can see that the stationary value of the biomass concentration is 0.5233 gr/l.

Based on the data in Table 3.2 and Figure 3.34, it was represented in Fig. 3.41 Biomass evolution, considering the origin of the coordinate axes point: t_0 = 260 min, x_0 = 0.3030 gr/l.

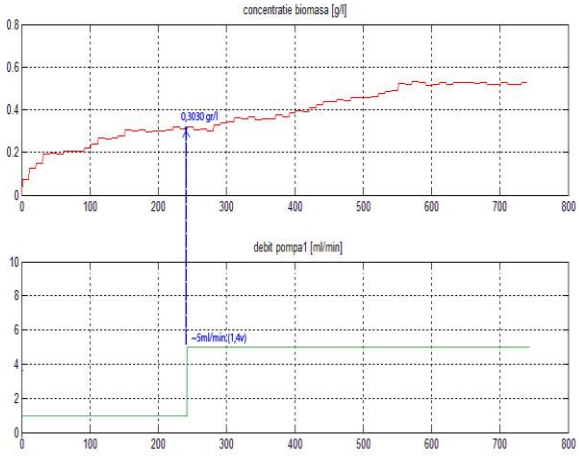


Fig. 3.34 - Evolution of the biomass concentration (gr / l)

Table 3.2: Biomass concentration determined for each image

1v=~2ml/min and 1.4v=~5ml/min

Im. Nr.	Val. Conc. [gr/l]	Cmd. p. [ml/min]	Im. Nr	Val. Conc. [gr/l]	Cmd. p. [ml/min]	Im. Nr.	Val. Conc. [gr/l]	Cmd. p. [ml/min]
1	0.0747	2	26	0.3030	5	51	0.4591	5
2	0.1235	2	27	0.3120	5	52	0.4613	5
3	0.1476	2	28	0.2991	5	53	0.4767	5
4	0.1932	2	29	0.3283	5	54	0.4870	5
5	0.1943	2	30	0.3379	5	55	0.4919	5
6	0.1926	2	31	0.3455	5	56	0.5220	5
7	0.2037	2	32	0.3617	5	57	0.5164	5
8	0.2078	2	33	0.3578	5	58	0.5307	5
9	0.2077	2	34	0.3649	5	59	0.5292	5
10	0.2212	2	35	0.3527	5	60	0.5142	5
11	0.2400	2	36	0.3587	5	61	0.5203	5
12	0.2661	2	37	0.3552	5	62	0.5258	5
13	0.2637	2	38	0.3764	5	63	0.5178	5
14	0.2671	2	39	0.3676	5	64	0.5268	5
15	0.2783	2	40	0.3843	5	65	0.5292	5
16	0.3064	2	41	0.3937	5	66	0.5260	5
17	0.3013	2	42	0.3924	5	67	0.5234	5
18	0.3073	2	43	0.4088	5	68	0.5270	5
19	0.2971	2	44	0.4255	5	69	0.5175	5
20	0.3000	2	45	0.4390	5	70	0.5189	5
21	0.3016	2	46	0.4368	5	71	0.5290	5
22	0.3069	2	47	0.4471	5	72	0.5184	5
23	0.3195	2	48	0.4431	5	73	0.5194	5
24	0.3103	2	49	0.4591	5	74	0.5298	5
25	0.3206	2	50	0.4575	5	75		

The response to the step signal of the process thus represented leads to the following conclusions:

1. the effect of the measurement noise is important, which is not surprising given the technique used for the assessment of biomass. In general, the noise level affecting biomass measurement in biotechnological processes is high;

2. the increase of the biomass concentration is approximately linear, which contradicts - to some extent - the technological considerations mentioned by the literature. Typically, if the experiment progresses normally, the time course of the concentration has an inflection point corresponding to the exponential growth of the cells;
3. after the approximate linear concentration variation is exhausted, the entry into the stationary phase occurs quite suddenly, with slight over-regulation, which is not common and can be a consequence of the effect of the measurement noise.

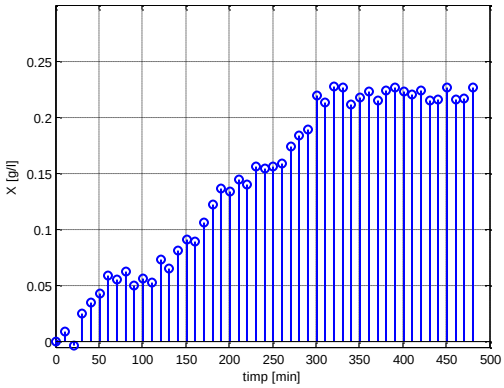


Fig. 3.41 - Evolution of biomass relative to origin t_0 = 260 min, x_0 = 0.3030 g/l

Using the experimental data available, classical identification procedures, ie the least squares method (matlab arx function) and the instrumental variables method (matlab iv4 function) do not allow us to achieve satisfactory results. Under these conditions, two heuristic solutions were tested in order to obtain a linear model whose response to the signal step satisfactorily approximates the experimentally obtained response (Annex 2- Program 2.1). The first solution is to obtain a linear model that achieves the approximation of the step signal response in Fig. 3.41, without imposing the requirement that this model be consistent with the usual processes revealed in bioprocessing modeling. Such a model has the transfer function

$$H_1(s) = \frac{K_1}{s}\left(1 - e^{-\tau s}\right)\frac{K_2}{T_1 s + 1} \tag{3.1}$$

in which K_1 = 0.2 / 30; K_2 = 1.1; t = 5 [h]; T_1 = 30 [min] - (Annex 2 - Program 2.2). Fig. 3.42 illustrates the quality of the approximation of the experimental response to that of the model (3.1). Even if a good approximation quality is obtained, the transfer function (3.1) has no support in bioprocessing modeling.

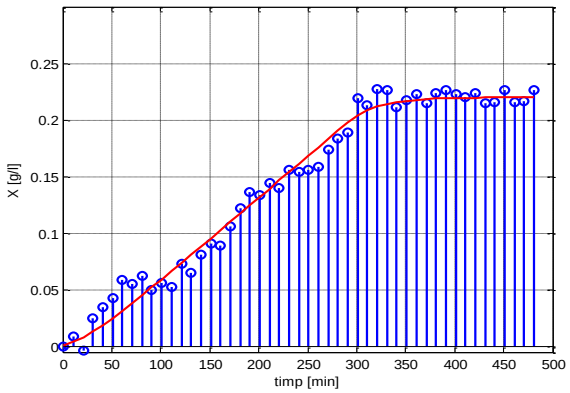


Fig. 3.42 - The answer of the model (3.1),in red,and the experimental response from Fig. 3.41

Consequently, it is preferable to look for a model that reflects the usual phases of bioprocess dynamics, even when noise can mask - to some extent - these phases.

A typical response is the inflection point corresponding to the exponential phase. The specific part reflected by the experimental data consists in the existence of a broad extension area, which corresponds to the vicinity of the inflection point. A model that can offer a near-linear variation area close to the inflection point in the step signal response is the multiple-pole model commonly used in the Strejc process identification method [76].Dacă s-ar utiliza funcţia de transfer:

$$H_2(s) = \frac{0.3}{(100s+1)^2} \tag{3.2}$$

for modeling the process, then the response of this system, y2 (t), together with the experimental response, yp (t), have the shapes in Fig. 3.43. In the mean area of the y2 (t) response the variation is close to the linear one, but the stabilization occurs at a higher value than the experimental stationary regime.

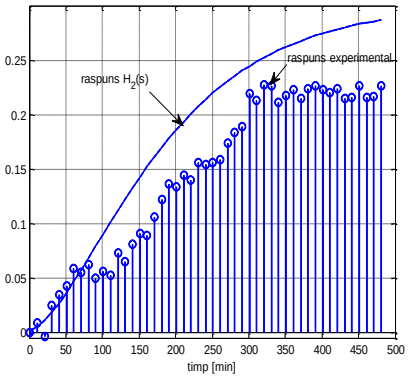


Fig. 3.43 - The experimental response and H2(s) system

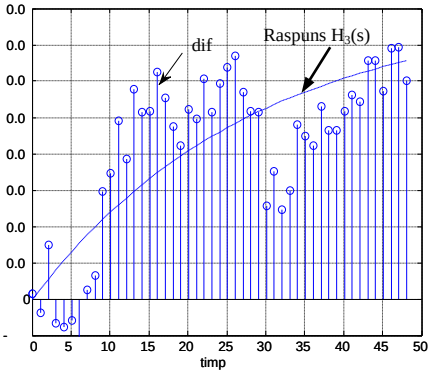


Fig. 3.44 - Difference dif and system response H3 (s)

Time evolution of the difference

$$dif(t) = y_2(t) - y_p(t) \tag{3.3}$$

shown in Fig. 3.44 by the Matlab stem instruction is approximated by the stepped signal response of the aperiodic element:

$$H_3(s) = \frac{0.08}{280s+1} \tag{3.4}$$

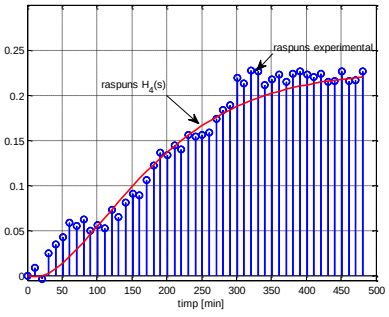


Fig. 3.45 -The experimental response and the H4(s) system

Under these conditions, the approximation of the experimental response is given by the relationship

$$\hat{y}_p(t) = y_2(t) - y_3(t) \tag{3.5}$$

where is the element's response to the transfer function. Consequently, the transfer function that approximates the process dynamics (at time scale in minutes) is:

$$H_4(s) = \frac{0.3}{(100s+1)^2} - \frac{0.08}{280s+1} = \frac{-800s^2 + 68s + 0.22}{2.8e6s^3 + 66000s^2 + 480s + 1} \quad (3.6)$$

From Fig. 3.45 it is found that this transfer function satisfactorily approximates the dynamics of the bioprocess and leads to an evolution of the infiltration point biomass concentration (Annex 2 - program 2.3).

3.7 Designing the controller for the biomass concentration regulation loop and its experimental validation

The design of the controller was done by frequent procedures. The Nyquist characteristics of the process, when using models (3.1) and (3.6), are represented in Fig. 3.46 with black and blue respectively. If the same PI controller with the transfer function is used for these process models

$$H_R(s) = K_R\left(1 + \frac{1}{T_{iR}s}\right) \quad (3.7)$$

in which KR = 1.8 and TiR = 50 [min], the Nyquist characteristics of open loops are given in Fig. 3.47. The amplification and phase margins are mdB = 2.44, γ = 380, using the model (3.1), respectively mdB = 6.1, γ = 40.70, when using the model (3.6).

In Fig. 3.48 it shows the system response in a closed loop with the PI controller having the above mentioned parameter values and the process transfer function H4 (s) - the relation (3.6), obtained with the simulink scheme in Annex 2 - Fig. A2.1.

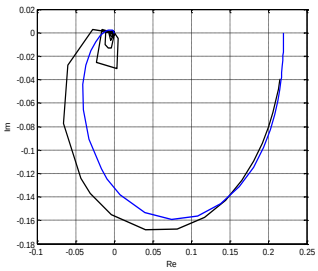


Fig. 3.46 - Nyquist characteristics of the process modeled with the transfer functions (3.1) (black) and (3.6) (blue)

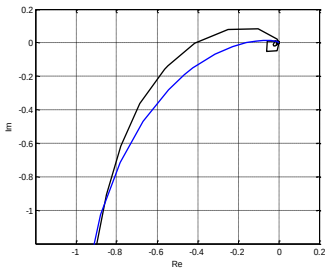


Fig. 3.47 - Nyquist characteristics of loops in open circuit using models (3.1) (black) and (3.6) (blue)

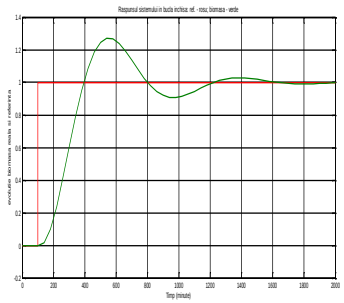


Fig. 3.48 – Closed loop system response

From an informational point of view, the implementation of the automatic regulating system of the yeast growth process uses two functions: the first function is the acquisition of the images and calculates the biomass concentration (practically implements the biomass concentration transducer), and the second function implements the PI controller.

The two functions communicate with each other via a txt file. It should be noted that the synchronization problems between the two functions have been solved so that the adjustment algorithm works correctly.

It also reads the reference from a txt file so that it can be changed in real time during an experiment. Taking into account a series of experiments previously carried out, an important detail was observed, namely that the direction of biomass evolution (increase or decrease) depends very much on the initial volume from which the experiment begins.

If a small volume (0.5 liter) of the bioreactor is used, and the substrate flow entering the bioreactor is high, biomass tends to decrease due to the fact that the inflow tends to dilute biomass from the bioreactor. However, there are many factors that lead to the emergence of exceptional cases, where biomass, having a large amount of food and having the remaining parameters at "optimal" values, can increase.

If a bioreactor with a relatively large volume (eg 5 liters) is used, if the substrate flow entering the bioreactor is high then the biomass concentration tends to increase because it will have a lot of food and the flow of the liquid at the inlet in the bioreactor will be small compared to the initial biomass volume, which makes it almost negligible. When the input flow rate is small, the trend is also increasing, but in a very long time, rather a stabilization at a certain value because the amount of food is small in relation to the number of cells it consumes, and the flow rate of the liquid entering with the substrate is negligible.

Experiment nr. 4:

Validation of the automated regulating system of yeast growth of S. cerevisiae was carried out in experiment no. 4. This was done in a closed loop, the KR and TiR parameters being those determined at the beginning of this section. Initially, it was started with a volume of 5 liters and a biomass concentration of 0.5 gr / l, followed by two reference steps of 0.4 and 0.6 gr / l respectively (the curve represented in green in Fig. 3.49). Table 3.3 presents the values of the biomass concentration [gr / l] and the substrate feed pump [V], the substrate with the concentration of 125 gr / l, determined for each image, values obtained during the experiment.

Table 3.3: The biomass concentration values [gr/l] and the output control to the substrate feed pump [ml/min] determined for each image

Nr. Im.	Val. Conc. [g/l]	Cmd. pompă [ml/min]	Nr. Im.	Val. Conc. [g/l]	Cmd. pompă [ml/min]	Nr. Im.	Val. Conc. [g/l]	Cmd. pompă [ml/ min]
1	0.5038	0	26	0.3854	0	51	0.5231	2.720
2	0.4953	0	27	0.4060	0	52	0.5368	2.774
3	0.5063	2.024	28	0.3837	0	53	0.5283	2.732
4	0.4930	0	29	0.3821	0	54	0.5374	2.792
5	0.5091	2.03	30	0.4117	0	55	0.5434	2.768
6	0.5097	0	31	0.3863	0	56	0.5518	2.762
7	0,5012	0	32	0.3938	0	57	0.5429	2.738
8	0,4901	0	33	0.4154	0	58	0.5567	2.798
9	0,5023	2.042	34	0.4208	2.612	59	0.5489	2.744
10	0,5064	0	35	0.4244	2.642	60	0.5527	2.798
11	0,4890	0	36	0.4470	2.684	61	0.5672	2.792
12	0,4851	0	37	0.4484	2.624	62	0.5528	2.738
13	0,4904	0	38	0.4570	2.666	63	0.5682	2.822
14	0,4815	0	39	0.4621	2.672	64	0.5794	2.756

15	0.4771	0	40	0.4667	2.690	65	0.5859	2.708
16	0.4692	0	41	0.4765	2.708	66	0.5930	2.684
17	0.4716	0	42	0.4632	2.702	67	0.6067	2.648
18	0.4615	0	43	0.4759	2.810	68	0.6139	2.582
19	0.4581	0	44	0.4879	2.792	69	0.5991	2.546
20	0.4498	0	45	0.5058	2.768	70	0.6033	2.612
21	0.4521	0	46	0.5189	2.714	71	0.5970	2.594
22	0.4350	0	47	0.5240	2.672	72	0.6030	2.624
23	0.4246	0	48	0.5180	2.672	73	0.5972	2.594
24	0.4174	0	49	0.5237	2.732	74	0.6032	2.624
25	0.4018	0	50	0.5301	2.726			

In Fig. 3.49a it can be seen that the system follows the setpoint, which means that both the biomass concentration transducer made on the basis of the image processing technique and the controller work correctly. In Fig. 3.49b, it is possible to observe the variation of the control value in flow units.

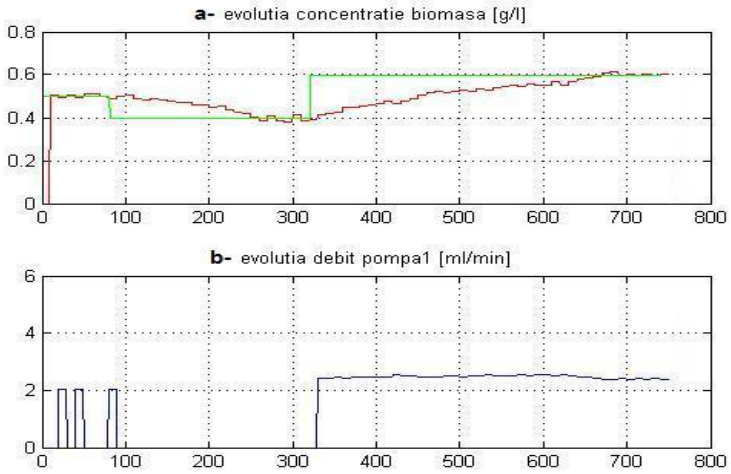


Fig. 3.49. Evolution of biomass concentration (a) and control value evolution (flow rate of substrate feed pump - b)

Chapter 4

Classification of the physiological state of cells using image processing techniques

4.1 Introduction

Equipment where biological reactions or intense transformations, such as bioreactors, occur, must ensure a non-limiting transfer of nutrients from the culture medium to the cells, as well as favorable conditions for their development.

The present paper aims to diagnose bioprocesses with a case study of *S. cerevisiae* yeast cultures, with an important personal contribution to the introduction of the notion of cell viability or the viability of cells.

This is a physiological feature of cell culture which is a major concern when referring to bioprocesses, this feature being directly associated with productivity. Frequently, cell viability is assessed by microscopy by mixing the suspensions with specific reagents that stain the entire cell or parts thereof. Thus, dead cells will become colored because the membrane is damaged and allows the absorption of the dye inside, while the living ones will remain uncolored, allowing visual discrimination between living cells and death. For example, dyes such as methylene blue, evans blue, tripanic blue, iodine propidium, neural red, phenosafranines [79], [80], [81] are used to assess the integrity of the cell membrane. Otherwise, a number of specific dyes, such as fluorescein, oxonol, carboxifluorescein diacetate, etc. [82] and [83] were used to assess the metabolic activity of the cells. These methods are invasive and sometimes the small number of cells in the sample is rather unrepresentative for the entire population.

Recently, the determination of cell viability through non-invasive imaging techniques was possible with dark field microscopy microscopes. In [84] a vector machine was used that used learning techniques to discriminate between living and dead cells. Two cultures of yeast Saccharomyces cerevisiae, the first formed only from living cells, the other only from dead cells, were used for this.

In Chapter 4 of the Ph.D. thesis, a first bioprocessing analysis was carried out using images captured with a 1.3MPx black-and-white Olympus camera in an experiment in which a first level of diagnosis was sought, which consists of identifying and classifying cells in two categories: living and death respectively. Validation of the results was performed using a contrast phase microscope (contrast phase microscopy). As this first level of diagnosis is not enough to diagnose the evolution of the cell population, a yeast cell grading algorithm was developed using images captured with a 1.3 mpx color camera in a second experiment that lasted 6 days. Because the amount of information contained in color images is superior to black and white images, it allowed a better classification of the physiological state of the cells (a refinement of their state), with five cell categories: young, mature, in division cells in the multiplication phase), old, dead. Validation of experimental results was accomplished through numerous laboratory tests performed simultaneously with image sampling.

4.2 Bioprocess diagnostic elements. Analysis of bioprocess evolution basedon physiological state of cell culture using black and white image processing techniques

In this section, the possibilities to determine biomass concentration and cell viability in yeast cultures were tested without using classical invasive techniques. At this stage, an algorithm has been developed with the following capabilities:

- to highlight viable cell features in images from a conventional light microscope. It is used to assist the operator when classifying viable and non-viable cell images.
- count the cells in the image and evaluate the biomass concentration.

4.2.1 Materials and methods

The biological agent used for this study was a *Saccharomyces cerevisiae* MIUG D9 culture strain from the college of the Faculty of Food Science and Engineering, "Dunărea de Jos" University of Galati. The cells were initially cultured on Scharlau agar malt extract (malt extract - 30 g / l, soybean peptone - 3 g / l, agar - 15 g / l), and taken up in saline when necessary. Images were purchased with a Olympus BX41 phase contrast microscope with a 1.3 MPx Olympus TV1X-2 monochrome camera. The imaging acquisition was performed with a 40x and 10x eyepiece, using both phase contrast microscope and bright field microscope. The light intensity between the two types of images required an adjustment: while in the brightness range of 2.5 (on a scale from 0 to 6) it is sufficient, phase contrast images require a level of 4 for to be visible. Images were purchased in grayscale and processed on a regular PC.

The study contains several sets of images taken from different cultures of the species *Saccharomyces cerevisiae*. The first images were taken on fresh cultures, nutritionally fed, followed by the study of old crops left without nutrients for a certain period of time. In all cases, the images were taken in two ways: using the Phase-contrast filter of the microscope and without using the filter (bright field microscopy).

Fig. 4.1 shows the 400x magnified image of a live cell and a dead cell taken in the phase contrast variant. The difference between a living cell and a dead cell can be easily noticed. The living cell has a well-formed membrane and the interior is bright, while the dead cell is darker indoors and is blurred / diffuse. Some of the dead cells also have fractures in the membrane and their intracellular matter is dispersed in the suspension. The cells of Fig. 4.2 are the same as those in Fig. 4.1, the only difference being the lack of phase contrast filter. The two cells can be distinguished, but the differences are much smaller compared to the situation where the phase contrast filter was applied on a microscope. This is not a simple task for an operator to discriminate in images, such as that in Fig. 4.2, between viable and dead cells.

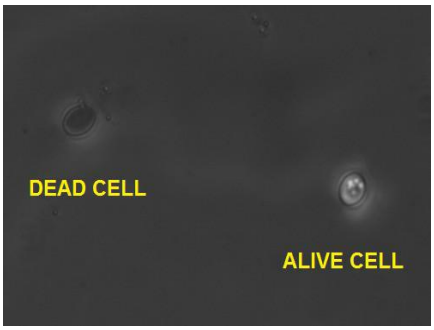


Fig. 4.1- Comparison between a living cell and a dead one one in phase-contrast microscopy

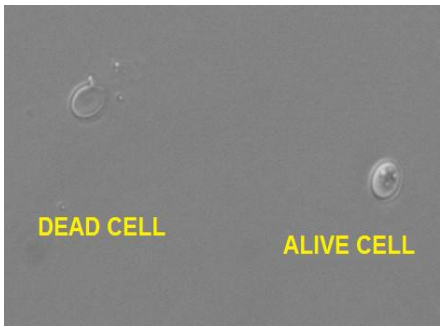
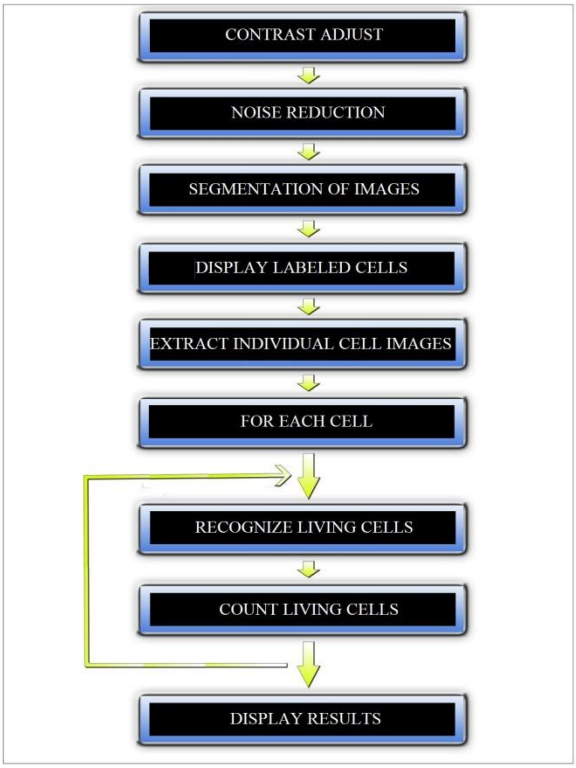


Fig. 4.2 - Comparison between a living cell and a dead one in light field microscopy

The cell-enhancing algorithm was developed on bright microscopic fields. This extends the applicability of the algorithm to laboratories that do not have a phase-contrast microscope and can help the operator assess cell viability and evaluate biomass concentration. In order to exploit the properties of images containing cells, the improvement of the method consists of the following operations: image contrast adjustment, noise reduction, image segmentation, cell recognition and labeling, live cell recognition and counting, biomass concentration determination.

4.2.2 The image processing algorithm

In the classical method with methylene blue, it is added as a dye and then the image is analyzed optically under a microscope. Healthy viable cells will remain uncolored while dead cells will be stained in blue due to membrane fractures.



On the contrary, compared to the classical method, the proposed algorithm improves the images of viable yeast cells without the use of methylene blue. It has been developed using a number of image processing functions and runs as a Matlab program that performs the following steps:

Step 1: *Adjust the contrast of the image*

Step 2: *Noise reduction*

Step 3: *Segmentation of images*

Step 4: *Display labeled cells*

Step 5: *Extract individual cell images*

Step 6: *Recognize living cells*

Step 7: *Count the living cells*

Fig. 4.3 - The block diagram of the black and white image processing algorithm

Depending on the result returned by the recognition function, the algorithm calculates and ndisplays the number of cells considered live in the Matlab command window.

4.2.3 Experimental results of the cell recognition algorithm (experiment no5)

Fig. 4.4 shows the original image that is displayed unchanged. After running the algorithm, a set of images was generated to illustrate the steps of the algorithm and the results obtained.

In Fig. 4.5 shows the same cells (Figure 4.4) in phase contrast microscopy for comparison. The next step is the contrast adjustment, which provides a much better contrast image (Figure 4.6). Fig. 4.7 shows the segmentation stage. The result is a binary image. In Fig. 4.8 cells in the filtered gray shade image are framed in red borders as determined on the segmented image in step 5. The biomass concentration (in percent) is also displayed. From the image shown in Fig. 4.8 it can be seen that each cell is extracted and saved separately as a standalone image for further analysis in order to determine whether it is alive or dead.

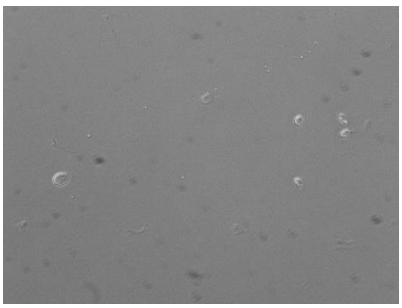


Fig. 4.4 - Image of yeast cells in bright field microscopy

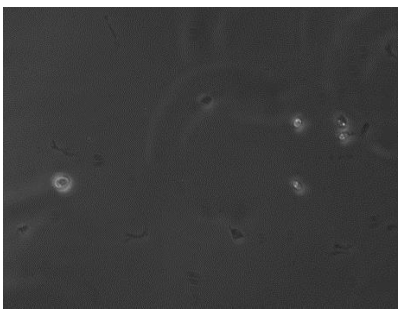


Fig. 4.5 - Image of yeast cells in phase contrast microscopy

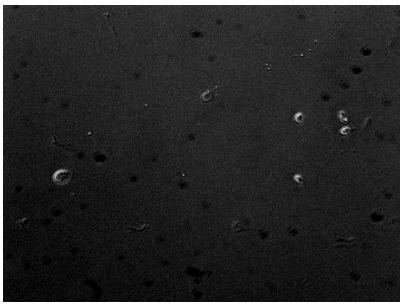


Fig. 4.6 -Image with adjusted contrast

By analyzing these images, each individual cell can be compared to the image taken with the phase contrast filter microscope, and thus the accuracy of the algorithm is validated. By repeating these experiments on multiple images, both with and without phase contrast filter, the average error rate for identifying cell viability can be estimated.

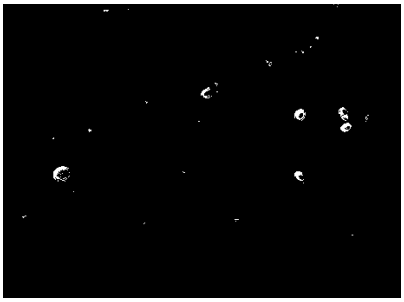


Fig. 4.7 – Segmentad image

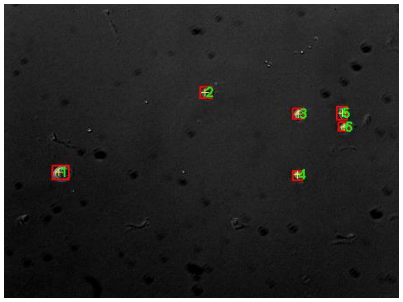


Fig. 4.8 - Identified cells (total cell number = 6, concentration = 0.32%)

Fig. 4.9 contains the marked individual cells, which are classified as viable or dead. The algorithm found a dead cell (second) and, compared to the original phase-shifter image, it can be concluded that this result is correct. The algorithm ran on a set of grayscale images taken with an Olympus microscope. As a consequence of the trials, several sets of results were obtained. The result of step 7 of the algorithm gave an error of around 20%. Validation was performed by a human operator who recognized live cells and dead cells on the same set of images but using a contrast filter.

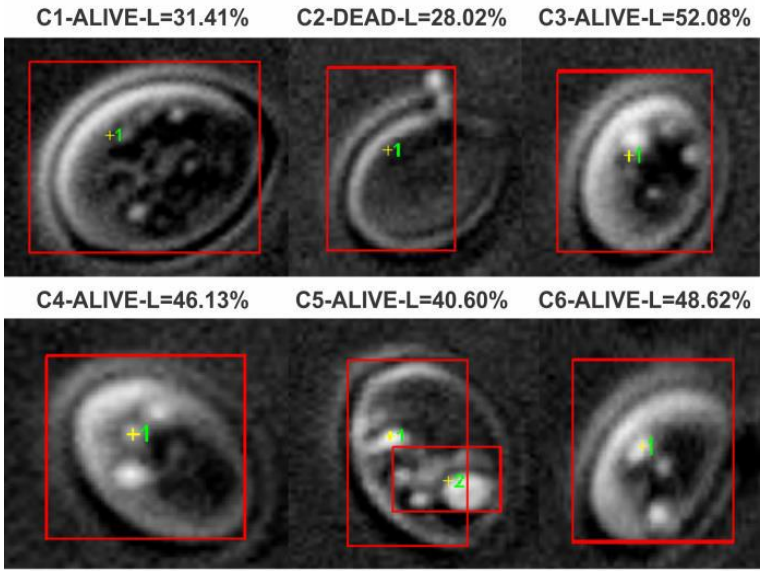


Fig. 4.9 - Individually labeled and classified cells

4.2.4 Study of identification errors

The main causes of errors are the large differences in brightness and contrast of the images taken at the microscope. These differences are due to several reasons, such as slate density, microscope focus, illumination, lamella characteristics (areas that allow more or less light to pass). Consequently, step 1 contains a brightness / contrast correction and the thresholds used in steps 3, 5 and 6 are determined experimentally, subject to changes for other species of microorganisms.

Another cause of errors is impurities, such as dust dots on the lens, scratches and other impurities of the suspension, or possible optical imperfections of the lamella. These can generate errors in cell detection and background differentiation. An example is the image shown in Fig. 4.10 which contains 5 cells and the algorithm detects only four.

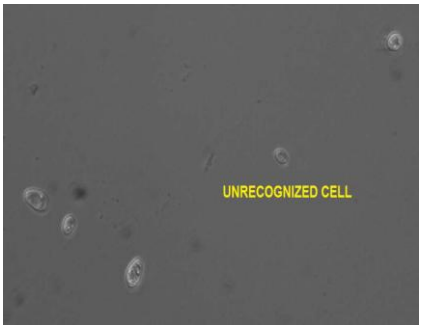


Fig. 4.10 - Image obtained in the light field contrast microscopy of a cell phase yeast culture

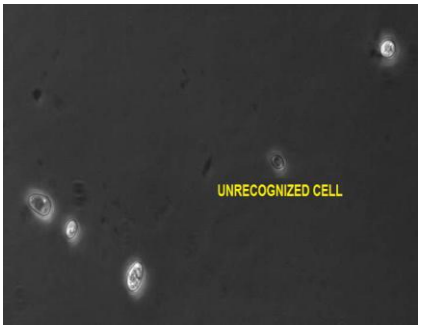


Fig. 4.11 - Image with phase filter of cells shows in Fig. 4.10

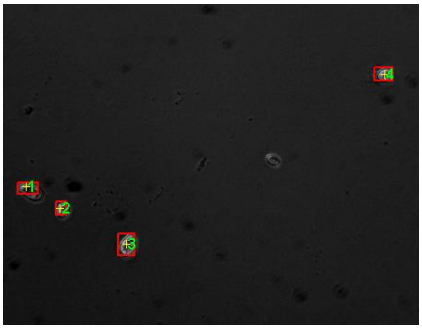


Fig. 4.12 - The result obtained by the algorithm with recognized and unrecognized cells

If the same picture is taken but taken up using the phase contrast filter of the microscope (Figure 4.11), we can see three viable cells, a "less viable" cell, and a dead cell may be seen. The dead cell was not detected by the algorithm due to the dust removal thresholds in the image. These thresholds are guided by the amount of impurities and their brightness. The impurities of Fig. 4.10 were successfully removed but with the cost of an unrecognized cell. It can also be observed that after the filtering, the impurities present in the image are less noticeable than in the original one. In Fig. 4.12 one can see a 5-cell image. On average, only one cell was labeled wrong, therefore the error rate is estimated at 20%. The algorithm ran on a set of eight images, containing a different number of yeast cells and also having another brightness and contrast.

4.2.5 Validation of results

In carrying out this study, the same yeast cells, of the species Saccharomyces Cerevisiae, were grown from laboratory cultures. Various samples were analyzed at certain time intervals to produce the image sets. To validate the visual recognition of the characteristics and differences between living cells and dead cells, an "aged" cell culture (Figure 4.14) was used in which both live and dead cells were identified.

In this figure, obtained through phase contrast microscopy, the live cells were indicated by the border. The culture in the original image (Figure 4.14) was left in the same position on the microscope slide for 5 minutes.

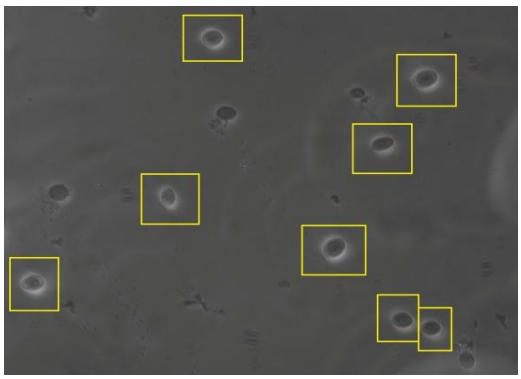


Fig. 4.14 - Live yeast cells (borded) from the initial culture

Due to the heat generated by the illumination of the microscope (halogen bulb illumination), the temperature of the suspension increased, generating unfavorable conditions for culture, which at that time caused the death and decomposition of cells. As a result, a new image was taken (Figure 4.15) where the characteristics of the same cells in the original image can be observed.

It was found that much of the initially living cells died within this time frame, displaying the same visual characteristics as those originally dead. At the same time, some dead cells have entered the decomposition phase, part of their internal content (nucleotides), spreading outward, along with the cytoplasm, due to breakdown of the cell membrane. In the initial figure, 8 living cells were identified and, after heat stress, only two living cells were identified, the remaining 6 cells died, some also entering the decomposition phase. Thus, the visual characteristics specific to the dead cells (color, lack of external aura on the outline), as well as those in the decomposition phase (the scattering of the cell contents outward) were validated.

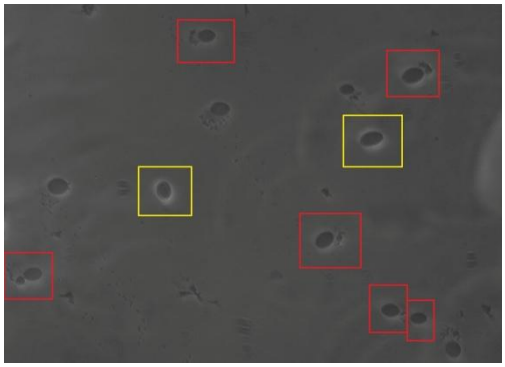


Fig. 4.15 - Yeast cells subjected to thermal stress (in the red border are cells initially living - now dead, in the yellow border are cells remaining alive due to heat stress)

4.2.6 Presentation of the processed image set

Fig 4.16 represents the initial image taken using the bright field microscopy technique.Fig. 4.17 is used to validate algorithm results. It can be seen that all the yeast cells in this image are alive due to the presence of the white aura around the cell margins and their well defined interior.

- First image:

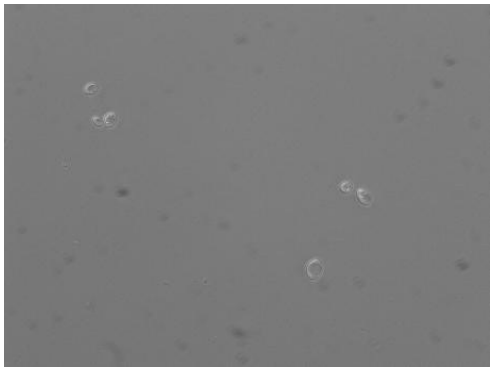


Fig. 4.16 - The initial image taken using the *bright field microscopy* technique

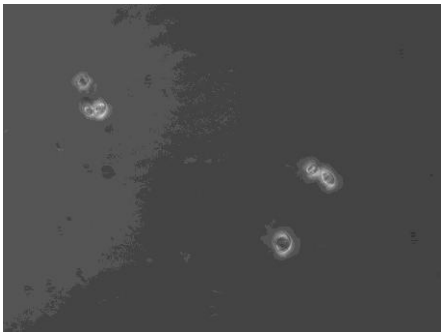


Fig. 4.17 The initial image taken using *contrast phase microscopy*

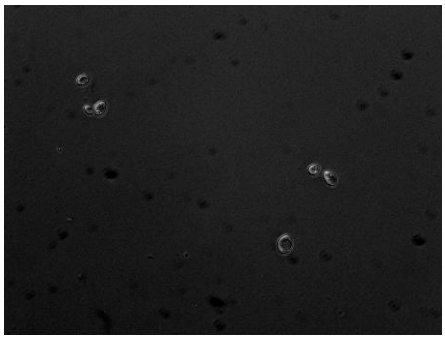


Fig. 4.18 - Image with adjusted contrast

Fig. 4.18 represents the result of the first stage of processing the algorithm to help correct cell identification and to distinguish background and impurities from them.

Fig. 4.19 represents the results from threshold segmentation.

Fig. 4.20 represents the result of applying the bwlabel function and shows the identified and labeled cells.

Fig. 4.21 shows a series of cells identified and classified from the processed image as well as the color coefficients calculated by the algorithm.

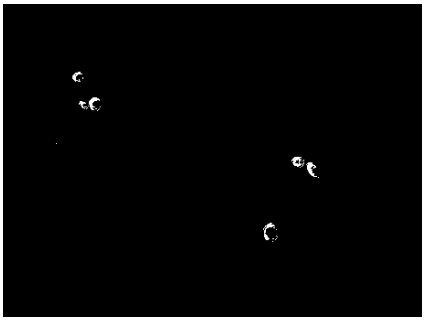


Fig. 4.19 -Image resulting fro segmentation

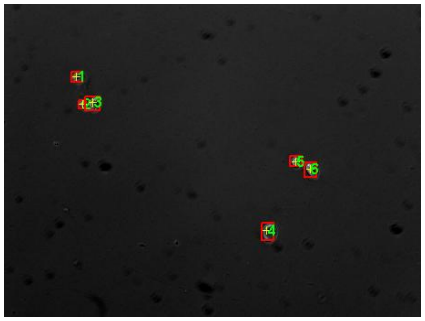


Fig. 4.20 - Cells identified (total number of cells = 6, concentration = 0.254%)

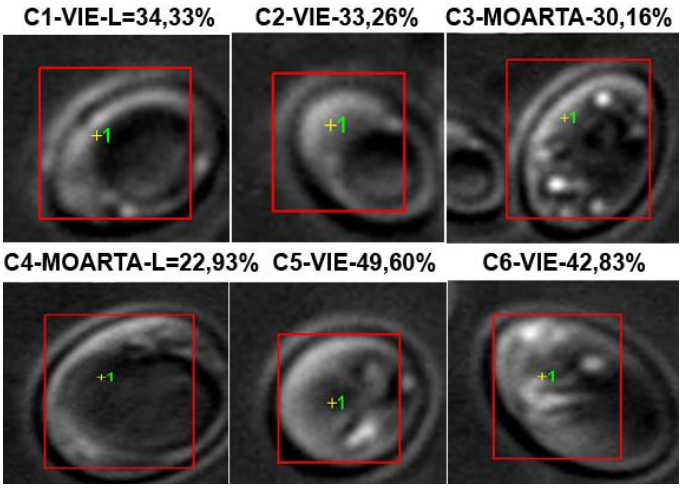


Fig. 4.21 - Individually identified and classified cells

For the error calculation, it can be seen that of the 6 identified cells, two are mistakenly classified as dead, so the error rate is 33%.

- The second image:

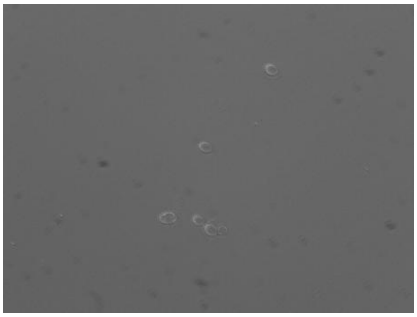


Fig. 4.22 - The initial image taken using *bright field microscopy* technique

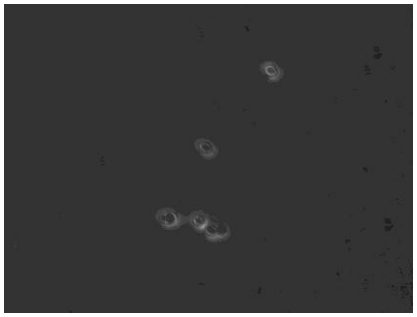


Fig. 4.23 The initial image taken using *contrast phase microscopy* technique

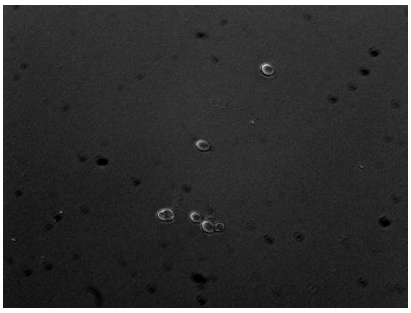


Fig. 4.24 - Image with adjusted contrast.

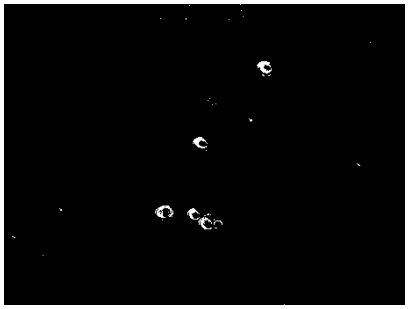


Fig. 4.25 - Image resulting from segmentation

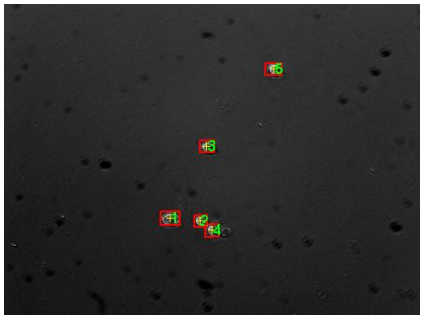


Fig. 4.26 – Identified cells (total number of cells = 5, concentration = 0.324%)

In this case, a detection error is also present. We can see 6 yeast cells in the image, and the algorithm recognizes only 5 of these, the sixth being considered impure and thus excluded. Of the 5 detected cells, only 4 were correctly classified.

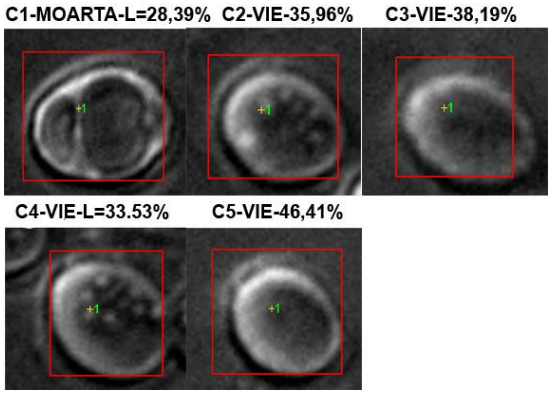


Fig. 4.27 - Individually identified and classified cells.

4.3 Evaluation algorithm for the development stage and classification based on information obtained from color image analysis

An experiment (**Experiment No. 5**) was carried out with the main purpose of determining the different stages of yeast culture evolution by analyzing the color images. For the study, all yeasts of Saccharomyces cerevisiae, pure culture on the standardized MEA (malt extract agar) medium, from the Microorganism Collection of the Bioaliment Research Platform of "Dunarea de Jos" University of Galati [90] were used.]

4.3.1 Materials and methods

Two experimental versions were performed: one on yeast extract glucose (YEG) medium with a favorable nutrient content required for yeast cell growth and multiplication, and the other on a auxotrophic G-free nitrogen medium (extract of yeast), having only the glucose source (glucose) required for basic cellular energy metabolism. For both media an inoculum of 1.76 • 106 ufc / ml (ufc - colony forming units) was used.

Five conditions have been determined in which cells can be found: young cells, mature cells, cells in the division, old cells and dead cells. To describe these states, two sets of parameters were used: Cell size (small, large, very large - corresponding to cells in the division). The second set of parameters is determined by the inner texture of the cells. It has been found that cells within which there are dark areas (due to elements called ergastic inclusions) are stress cells or, in other words, non-viable, unproductive cells.

Cell classification criteria have been developed on the basis of several laboratory analyzes, such as:

- optical density measurement (DO600) using the Hach Lange DR3900 Spectrophotometer (Figure A1.23, Appendix 1);
- cell count in Petri dishes with standard agar (SPC);
- measurement of nitrogen and glucose concentration.

All laboratory analyzes were performed daily, at the same time as the sampling of cell cultures.

To reduce statistical errors, the method of inoculating two Petri dishes in each decimal dilution was used in parallel and the arithmetic mean of the highest dilution plate was

calculated in which the number of colonies per plate did not exceed 300. The result, viable cells of the S. cerevisiae species (N), was expressed in μg / ml according to the formula:

$$N_{(ufc/ml)} = \frac{(n1 + n2)}{2 \bullet d} \quad (4.5)$$

where n1 and n2 represent the number of colonies in the two plates in which the count was made, and d is the dilution factor.

A microscope equipped with a color digital camera with a resolution of 1.3 mpx was used to capture images. The images were purchased using a 60X lens and a color camera with a magnification factor equivalent to a 10X eyepiece. The light intensity of the microscope was set to its maximum value and remained unchanged during the experiment. Images were purchased in RGB mode and processed with a regular computer. Images were captured using Saccharomices Cerevisiae yeasts.

The first medium was a growth-enhancing medium containing all the necessary nutrients (YEG - yeast extreme glucose). The second was a nutrient-free (G-glucose medium) nitrogen-free medium and containing a higher glucose concentration to see how yeast reacts in an unfavorable environment. Both media were inoculated under the same sterile conditions (standardized inoculation was performed using a Thoma cytometry). For culture, a Medline SI-300R shaker (Fig. A1.24, Appendix 1) was used with a set temperature of 25 ° C and a speed of 150 rpm. The experiment lasted 6 days, during which the images were taken daily using the two media (Figure A1.25, Appendix 1). On each microscope slide, the lens was moved to 30 to 50 points so that the images purchased were representative of the entire surface of the lamella.

Fig. 4.29 shows a comparison between different stages of evolution of the same crop. The images were taken on different days and captured with a magnification factor of 600X. Differences between cells can be easily observed. It can be seen that end-of-life or stressed cells, such as lack of nutrients, develop a dark inside structure, and rarely the membrane becomes irregular, not well-contoured.

The cells in this state are no longer productive, approaching the end of life, some of which are already dead, which can be seen in Fig. 4.29 and Fig. 4.30. This latency was confirmed by all laboratory tests performed at the same time as capturing images.

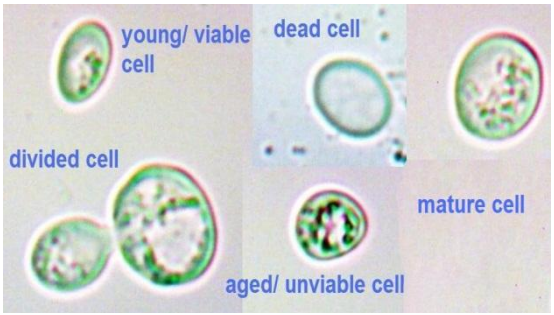


Fig. 4.29. Comparison between different stages of yeast cell evolution

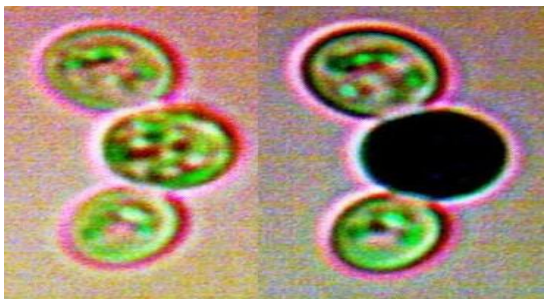


Fig. 4.30 - Old / dead cells and the same cells stained with methylen blue

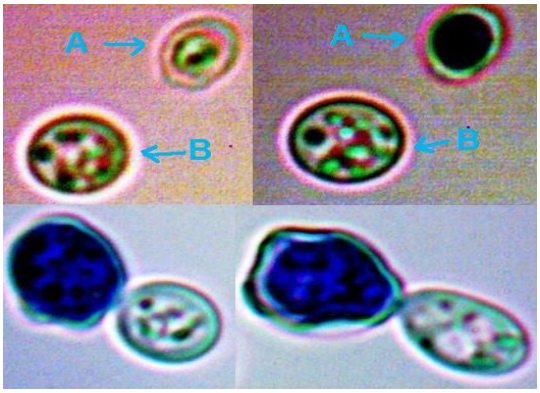


Fig. 4.31. A - Dead cell (normal + methylen blue), B - Old cell (normal + methylen blue). Two dead cells stained with methylen blue

In the first line of Fig. 4.31 there are two types of cells: one (A) showing an irregular membrane, which in this case was found to be dead following the use of the blue-blue method and the other, (B) - the old cell that turned out not to be still dead. In the second line there are two different cells with changes in the membrane structure of the same type with the first line cell (A), which have been found to be dead using the blue-staining method.
Both the experiments and the image processing algorithm using color images were also exposed in [92], indexed by ISI Proceedings and IEEE-Xplore.

4.3.2 Image processing algorithm

The classification algorithm is based on our research and uses the images obtained with an ordinary microscope and a color camera. The main purpose is to be able to recognize viable cells as compared to the old ones (unproductive) and, secondarily, to classify the stages of cell evolution, such as: young, in division, mature, old, dying. In order to achieve the proposed goals, the algorithm has the following objectives:

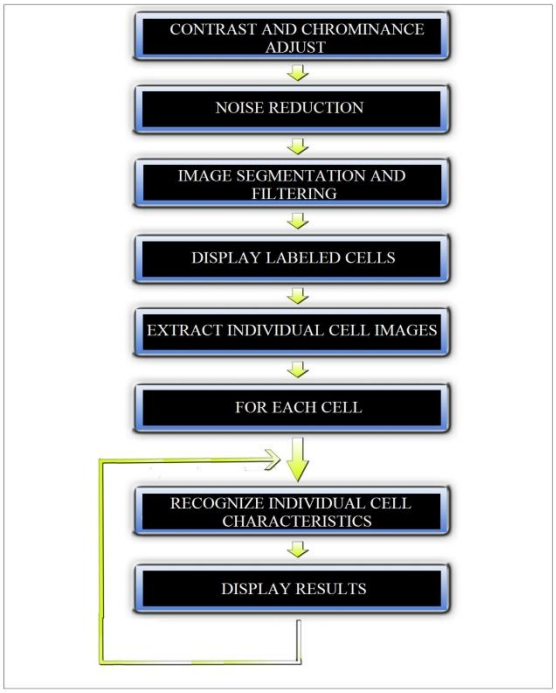
- adjusting the luminance and chrominance of the color image;
- reduction of noise;
- image segmentation;
- display labeled cells
- extracting individual cell images;
- recognizing the characteristics of each cell.

The objectives of the proposed algorithm are:

1. Analyze a series of microscopic images to extract images of individual cells;
2. Improvement of the relevant graphical properties of cell images in order to help the operator recognize the status of each cell;
3. Extracting cell characteristics, which are then useful for automated cell viability classification.

This algorithm has been developed from the previous version and uses new image processing methods, according to the new final objectives. It was tested using the Matlab environment and its corresponding functions. The algorithm is shown in Fig. 4.32.

At this stage, cells are classified. For this purpose, individual images of previously saved cells are converted into grayscale images. Using k-means clustering, new images of individual cells are obtained. They are subject to a contrast adjustment. The cell images are first segmented and new images are obtained, from which the contour, area, and brightness.



Step 1: Adjust the contrast and chrominance of the image
Step 2: Noise reduction
Step 3: Segment and filter images
Step 4: Display labeled cells
Step 5: Extract individual cell images
Step 6: Recognize individual cell characteristics

At this stage, cells are classified. For this purpose, individual images of previously saved cells are converted into grayscale images. Using k-means clustering, new images of individual cells are obtained. They are subject to a contrast adjustment. The cell images are first segmented and new images are obtained, from which the contour, area, and brightness.

Fig. 4.32 - The block diagram of the color image processing algorithm

The threshold was experimentally set at 0.3 and the minimum area of 30000 pixels. The ratio between the cumulative area of detected nucleotides and the surface of the whole cell represents the final characteristic, named as *ratio*:

$$ratio = \frac{\sum patterns\ area}{cell\ area} \tag{4.14}$$

This feature is useful for classifying cells as young, mature, old and dead. The classification conditions are as follows: if the cell surface is less than 8500 pixels, then the cell is young, otherwise it is classified as either the division cell (more than 10000 pixels) or mature (between the two values).

Experimentally, by visual analysis of multiple cell images, it was found that if the ratio is less than 5%, the cell is dead, if it is greater than 15%, the cell is old and between 5% and 15% , may be young or mature, depending on the cell's area.

The following graphs present the laboratory analyzes performed during the experiment: optical density (Fig 4.67) in the two media, count of cell colonies in the two media (Fig. 4.68), glucose concentration in the two media and nitrogen concentration. One can notice the entry into the stationary state of cells in the YEG environment on the third day, respectively, of the G environment on days 3-4, slower due to adaptation to the unfavorable environment.

It can be seen in Fig. 4.69 that on day 4 the glucose concentration increases in the YEG medium because after reaching the maximum biomass growth point on day 3, a biomass concentration decreasing phase (cells die and decompose) releases back some of the nutrients .

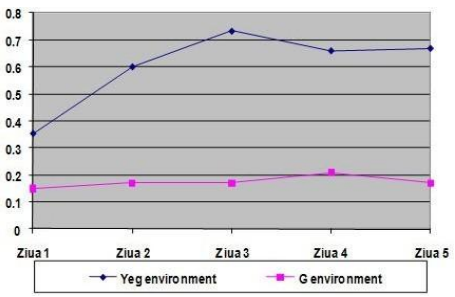


Fig. 4.67-The evolution of optical

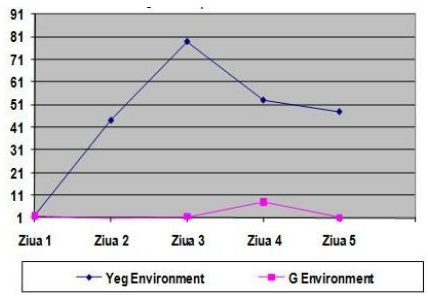
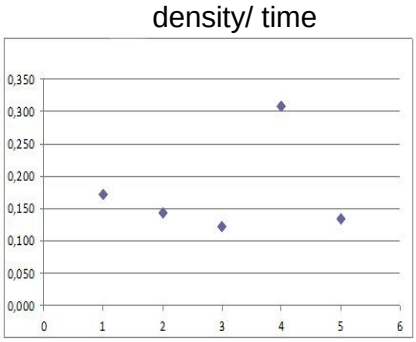


Fig. 4.68 - Evolution in time of counting



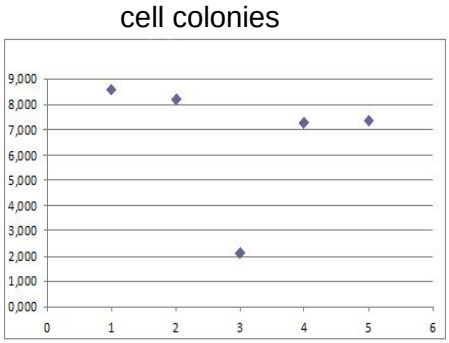


Fig. 4.69 - Conc. glucose [g / l]-YEG medium **Fig. 4.70**-Conc. glucose [g / l]-G medium

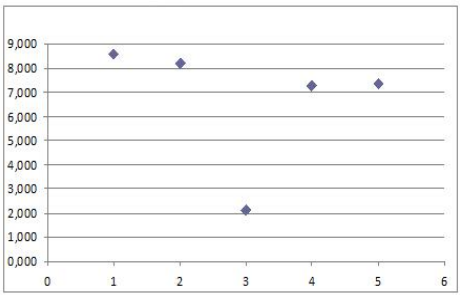


Fig. 4.71 - Nitrogen concentration [mg/l] - YEG medium

It can be seen in Fig. 4.69 that on day 4 the glucose concentration increases in the YEG medium because after reaching the maximum biomass growth point on day 3, a biomass concentration decreasing phase (cells die and decompose) releases back some of the nutrients . Fig. 4.70 shows the evolution of glucose concentration in G medium (low in nutrients). There is a decrease in day 3 concentration due to the consumption of glucose by the growing population, and on day 4 the maximum growth point will be reached. Fig. 4.71 shows the evolution of nitrogen concentration in the favorable medium (YEG). It is noted that the cells in this medium consumed more nitrogen and less glucose, unlike the G-cells that consumed the highest nutrient, glucose. As a result, on the day of reaching the maximum growth point (Day 3), it can be seen that the amount of nitrogen concentration drops to the lowest point, and then it increases due to the decomposition of the cells.

4.3.3 Results obtained with the image processing algorithm

After running the algorithm on an image, the following parameters are displayed for each individual cell: cell area, number of dark areas, ratio of area of dark areas to cell area. The following is an example of detection for each cell type (Figures 4.72-4.76).

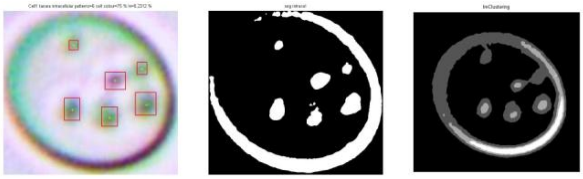


Fig. 4.72 - Recognizing a young cell (patterns = 6, area = 8490, ratio= 5.23)

Fig 4.73 - Recognizing a mature cell (patterns =10, area = 17181, ratio=6,52)

Fig. 4.74 - Recognizing a cell in division stage (patterns = 3, area = 21941, ratio = 5,91)

Fig. 4.75 - Recognizing an aging cell (patterns = 57, area = 9135, ratio = 27,29)

Fig. 4.76 - Recognizing a dead cell (patterns = 2, area = 12352, ratio = 1,82)

The first column contains the images of the color cells as they are present in the original image and the internal nucleotides detected, the second shows the images resulting from the segmentation, and the third presents the images resulting after the clustering method and the internal cellular detection of the algorithm image processing. Studying the resulting images, it can be seen that they favor recognition by the human operator, but at the same time the characteristics extracted by the algorithm are sufficiently relevant in an automated classification procedure. An example of running the algorithm on an image taken by the color camera on the third day of the experiment, as well as the images resulting from the processing, are presented below.

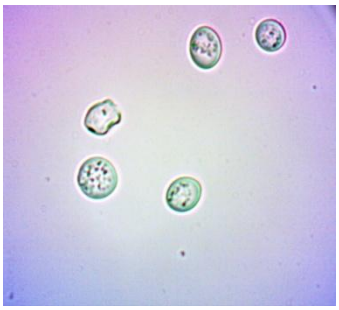


Fig. 4.77 - Original captured image by the microscope camera

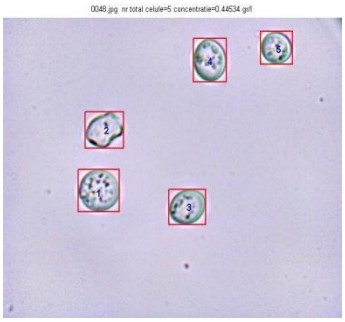


Fig. 4.78 The best quality image resulting from image processing

In Fig. 4.77 is the captured image and in Fig. 4.78 is the image with the best qualities resulting from the processing (Step 1), segmentation and labeling of the identified cells (Step 2 - Step 4). Figures 4.79 - 4.83 present the five cells identified in series of three images. In the first image on the left one can see the internal nucleotide recognition of the cell, the middle image shows the cut cell in the image on which it is calculated and the area and in the image on the right is the cell resulting from the application of the K-means Clustering method. Several cell contour detection techniques have been tested:

- canny method;

- hough method;
- entropy method;
- watershed method;
- threshold segmentation method (matlab);
- K-means clustering.

Following tests performed on numerous cell images, it was experimentally found that the K-mean method provides the best results in detecting individual cell contours. It can be seen from Fig. 4.79 - 4.83 that a dead cell and four mature cells were determined according to the parameters that are displayed for each cell.

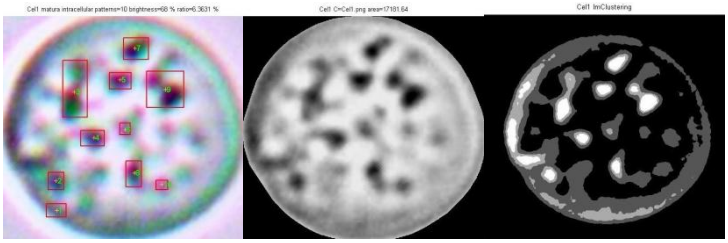


Fig. 4.79 - Cell 1 (mature, patterns =10, area = 17181, ratio = 6,3%)

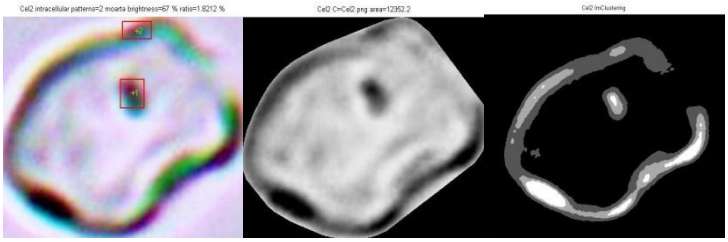


Fig. 4.80 - Cell 2 (dead, patterns = 2, area = 12352, ratio = 1,8%)

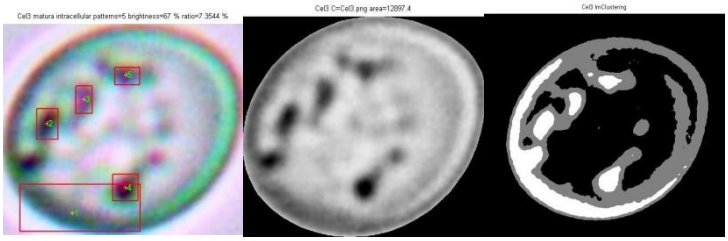


Fig. 4.81 - Cell 3 (mature, patterns = 5, area =12897, ratio = 7,3%)

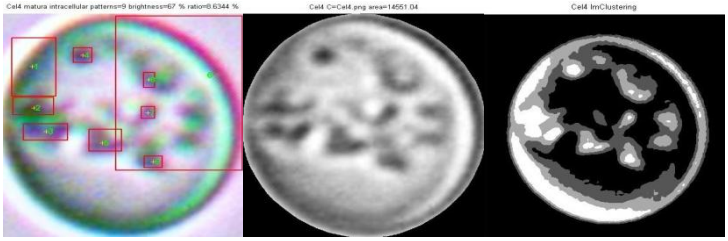


Fig. 4.82 - Cell 4 (mature, patterns = 9, area =14551, ratio = 8,6%)

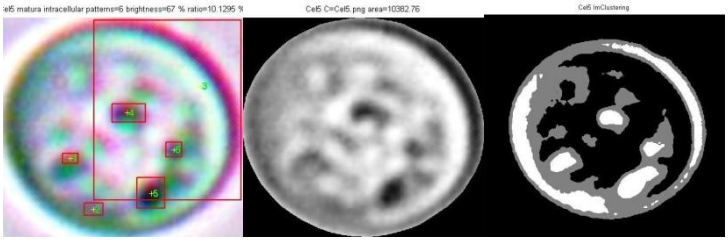


Fig. 4.83 - Cell 5 (mature, patterns =6, area =10382, ratio =10,1%)

4.3.4 Study of algorithm errors

During the testing of the algorithm a number of identification and classification errors were observed. In Fig. 4.84 it can be seen as an error of identification that the chick of cell no. 2 is not identified because it is of a very small size and is classified by the algorithm as impure and therefore excluded. In Fig. 4.85 one can distinguish the same error in the previous case when a cell in the division is not recognized due to the very small size of the chicken. Another type of error present can be seen in the picture in Fig. 4.86 where cell no. 2 is not recognized as the cell in the division, but two different cells are recognized. This error is due to focusing the microscope and the camera by producing a white blur between the two cells. This blur is cataloged by the algorithm as background and therefore distinguishes two independent cells.

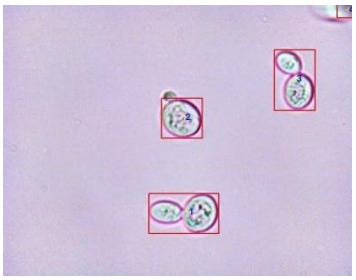


Fig. 4.84 Cell identification error

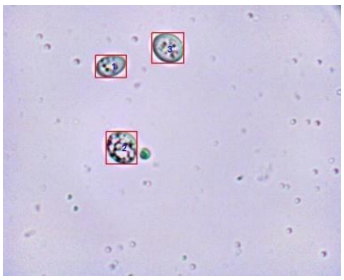


Fig. 4.85 - Cell identification error

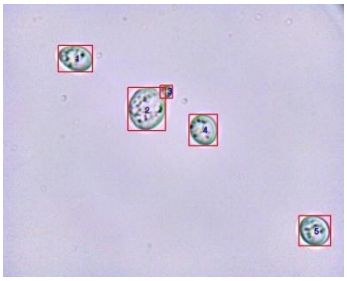


Fig. 4.86 - Cell identification error

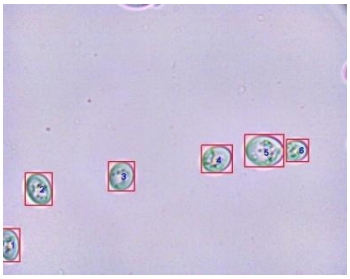


Fig. 4.87 – Image without identification errors

In Fig. 4.87 no yeast cell identification errors have occurred, so it can be said that for this picture the error is 0%. The following figure (Figure 4.88) may encounter a classification error. Cell number 7 is a dead cell with no intracellular content but, due to a partial recognition of it, is classified as incomplete recognition (without the left membrane). Another type of classification error is to classify a cell as an old cell when, in fact, it is mature. This type of error occurs in blurred / blurred images where the cellular interior is not well-highlighted. The algorithm was run on a set of ten images, containing a different number of yeast cells in different stages of evolution. Table 4.1 and Fig. 4.89 shows the synthetic results.

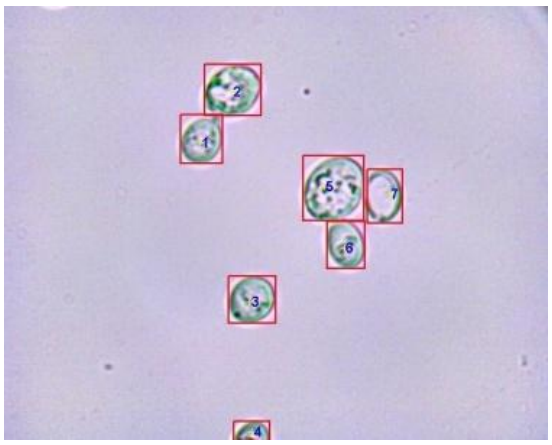


Fig. 4.88 - – Image without identification errors, but with clasification errors

In Fig. 4.87 - cell number 1, as well as Fig. 4.88 - cell number 4, are at the edge of the observation field, so they are partially viewed. In these cases, the algorithms compare the area of these partial cells with the minimum area (the threshold for recognition between an impurity and a cell), and if the area is greater than this threshold, the object is considered the cell and used in the next steps. The cell area is also taken into account when determining the biomass concentration.

Table 4.1 The average error introduced by the cell recognition algorithm

Imag.No.	1	2	3	4	5	6	7	8	9	10
Erorr	30%	30%	20%	0%	15%	7%	20%	0%	25%	15%

Final conclusions

Bioprocesses are processes that involve the development of microorganisms cultures for the purpose of naturally producing products of interest to human communities, such as medicines, pigments, biofuels, enzymes etc. Waste water treatment processes, where biotechnologies are the foundation of these processes, must also be mentioned. If at first biotechnology developed as a stand-alone science with more or less significant results, after 1980 biotechnology specialists were mainly focused on using automation techniques to streamline these processes. In fact, we can speak of an interdisciplinary field where contribute biotechnology and automation, to which were added computer science, mathematics and other sciences (physics, chemistry etc.). We can assert that the field of biotechnological processes has become a challenge for the specialists in control who have found a "technological niche" for developing and applying more and more advanced modeling techniques and control algorithms for making bioprocessing more efficient.

A delicate problem in controlling bioprocesses is that of measuring the main variables of interest, designing control loops or diagnosing bioprocesses. There are variables (eg concentration of biomass, substrate, metabolic products etc.) that cannot be measured on-line simply because there are no sufficiently precise, reliable sensors and at an acceptable cost price to allow their direct use in automation equipment. Using state estimators can be a solution to overcome this difficulty, provided that a mathematical model of bioprocessing is available.

In essence, this PhD thesis proposes a new method for measuring the magnitude of interest in bioprocesses in order to implement control algorithms or to diagnose them. The method is based on image processing techniques, for which a technical solution for the measurement of some bioprocess sizes, proposed in a case study, is proposed to increase yeast of the *S. cerevisiae* species. Thus, using the image processing technique, a method for measuring the biomass concentration was made. The process consists in automatically counting cells in a picture obtained using a classic microscope and a 1.3 mpx color camera.

Also, a biomass concentration measurement algorithm has been developed that analyzes color or black and white images, evens photometric parameters in the image, identifies and separates background cells and impurities, then counts, measures cells and calculates biomass concentration. Validation of the method was performed by comparison with measurements performed using a classical method, determination of dry matter in culture.

A communication interface has been designed and built between the automation equipment of a classic bioreactor and a purchase card connected to a PC. Through this interface, which includes both hardware and software, the automation of a classic bioreactor has been carried out and experimentation has been validated on the operation of the "transducer" based on image processing techniques. The two software-related algorithms mentioned above are for controlling the growth process of *S. cerevisiae* yeasts and for image analysis. For this purpose, the bioreactor has been provided with a sensor that allows real-time sampling, thus closing a biomass concentration control loop. The designed sensor consists of a bypass, a classic microscope equipped with a 1.3 mpx color camera and a PC computer, equipped with the appropriate software, in this case the Matlab environment. The circulation of the suspension in the flow chamber was accomplished by means of peristaltic pumps, used as execution elements. As mentioned above, the automated system developed in the doctoral thesis was designed and validated in the case of the growth of yeast cultures of Saccharomyces cerevisiae, but it can be easily adapted and used for other cell cultures similar to yeast cells.

Because this system does not require surveillance or control from the human factor, it can be used for long-term experiments.

On the diagnostic side, a series of image processing algorithms for yeast cell recognition have been developed and can be used by an operator to analyze cell cultures. This is of real use to the biotech operator who can be warned about the viability of the micro-organism culture (if the culture has been contaminated, if the biomass develops in good conditions, the average age of the culture can be appreciated etc.) in order to decide to continue or stop the process with the idea of rescuing material and energy resources that would be consumed unnecessarily if the process would evolve towards a state of failure. The first algorithm is able to recognize whether a yeast cell is alive or dead using a regular light field microscope and a black and white camera with a precision of over 80%.

The cell classification method was validated using a phase contrast microscope. The second algorithm allows the recognition of individual intracellular characteristics based on which cells are classified into five categories: young, mature, divisive, old and dead. The algorithm uses color images and the *k*-Means Clustering method to determine the intracellular constitution of each detected cell. Validation of the method was carried out using an experiment on a yeast culture lasting 6 days. In the experiment, the following measurements were measured: biomass concentration by classical method (cell count in Petri dishes - SPC), optical density, nitrogen and glucose concentration measurement. In order to compare the results obtained with the mentioned algorithms, daily laboratory analyzes were performed in parallel with the capture of images.

Original contributions of the PhD thesis

In summary, the following contributions should be mentioned in the doctoral thesis:

1. Development and implementation of an image-based image transducer for measuring the bioprocessing interest rates with the two components: hardware and software (in the case of this doctoral thesis - biomass concentration).
2. Experimental validation of the transducer operation for the measurement of biomass concentration by image processing techniques in the case of a yeast growth process of Saccharomyces cerevisiae.
3. Making a flow and bypass cell, a component of the aforementioned transducer, to take culture samples for measurement; it should be noted that this flow cell is one of the most important contributions of the thesis, being the essential element of the transducer, determining the accuracy of measurement of the biomass concentration. In the thesis, the flow cell has been improved by successive versions, to achieve the performance required for automatic operation, as well as the optical characteristics that influence the accuracy of cell identification and classification.
4. Determination of the dynamic characteristics of the transducer for the measurement of biomass concentration by image processing techniques in order to establish the minimum sampling period.
5. Creating a cell detection algorithm and calculating the real-time biomass concentration by image analysis techniques.
6. Making an algorithm that evens photometric parameters from images and which is based on 2D discrete cosine transformation. Since the luminance and chrominance of images depend on several factors such as: the density of the suspension, the optical characteristics of the lens, the focus of the camera, the brightness of the lamp and the position of the lens on the exposure area, this algorithm automatically adjusts the luminance and chrominance of the color images differences are reduced and image properties are uniform.
7. Making a method for automatically determining the segmentation threshold of an image that obtains the smallest errors on a large number of images.
8. Identification of the yeast growth process of *Saccharomyces cerevisiae*, in two variants, the second variant (based on Strejc models) having a technological support.
9. Design of a linear controller for the biomass concentration control and thus validated the image-based transducer developed in the doctoral thesis.

10. Development and implementation of a control system including the biomass concentration transducer using image processing techniques.
11. Making an interface between the bioreactor equipment and a leading PC computer where both image processing algorithms and the bioreactor control algorithm are implemented.
12. Experimental validation of the controlled system, including the biomass concentration transducer using image processing techniques, on a yeast growth bioprocess of Saccharomyces cerevisiae.
13. Study of physiological elements characteristic of the yeast cells (from a biotechnological perspective), in order to extract their traits, which were later used in the classification of cells.
14. Making an algorithm for classifying the cell state from a two-class culture (living or dead) by black and white image analysis and experimental validation of the algorithm using phase contrast microscopy.
15. Making a cell classification algorithm in 5 categories (young, mature, in division, old and dead) according to certain parameters determined by intracellular constitution analysis, by color image analysis techniques, in order to diagnose the bioprocess.

Future research directions

Starting from the results obtained within the PhD thesis, the researches carried out can be continued in the following directions:

- Expanding the method of determining the biomass concentration and other cell species similar to those of the *Saccharomyces cerevisiae* species (whose form is quasi-spherical) by modifying parameters such as cell size, detection thresholds etc.
- In the case of the cell classification algorithm, it can also be extended to other species that have similar intracellular characteristics by observing internal cell transformations during their development.
- The interface proposed in this paper can easily be adapted for a field bioreactor and control equipment with different characteristics (volume, thermal inertia, different flows of peristaltic pumps etc.).
- Investigating the use of a color camera with a sensor resolution of more than 1.3 mpx, resulting in greater detection accuracy and lower errors.

Dissemination of results

The results of the research in the doctoral studies were presented in the following published articles:

1. **Laurențiu Marius Baicu**, George Ifrim, Laurențiu Frangu, Sergiu Caraman, ”*Viability diagnosis in biotechnological cultures through image processing*”, 19th International Conference on System Theory Control and Computing (ICSTCC), oct. 2015, Cheile Gradistei, Romania, pp 770-775 **- ISI Proceedings - IEEE**;
2. Ifrim, George, Titică, Mariana, **Baicu Laurențiu Marius**, Caraman, Sergiu, “*Dynamic Modeling of the pH in Lactic Acid Fermentation Processes*”, 19th International Conference on System Theory Control and Computing (ICSTCC), oct. 2015, Cheile Grădiștei, Romania, pp. 225-230 **- ISI Proceedings - IEEE**;
3. **Baicu Laurențiu Marius**, Caraman Sergiu, “*Estimating the viability of algae cells through image processing*”, Scientific Conference of Doctoral Schools from “*Dunărea de Jos*” University of Galați, Third Edition - Galaţi, 4-5 June 2015, poster;
4. **Baicu Laurențiu Marius**, Caraman Sergiu, “*Evolution analysis of yeast cells using advanced image processing methods*”, Scientific Conference of Doctoral Schools from “*Dunărea de Jos*” University of Galați, Fourth Edition - Galati, 2-3 June 2016, poster;

5. **Laurențiu Baicu**, George Ifrim, Vasilica Barbu, Laurențiu Frangu, Sergiu Caraman, "*Stage evaluation of cell growth in yeast culture through image processing*", 20th International Conference on System Theory, Control and Computing (ICSTCC), October 2016, Sinaia, Romania, pp.704-709 **- ISI Proceedings - IEEE**;
6. **Laurenţiu Baicu**, Laurenţiu Frangu, George Ifrim, Sergiu Caraman, "*Control of the Yeast Growth Process Using an Image Processing-Based Transducer*", 21st International Conference on System Theory, Control and Computing (ICSTCC), October 19 - 21, 2017, Sinaia, Romania **- ISI Proceedings - IEEE**;
7. **Laurentiu Baicu**, Sergiu Caraman, Laurentiu Frangu, Mihaela Miron, "*Measurement of the biomass concentration from a bioprocess by image processing techniques"*, The 5th International Symposium On Electrical And Electronics Engineering (ISEEE), 20 - 22 October, Galati Romania **– BDI – IEEE**.
8. Nicusor Nistor, **Laurenţiu Baicu**, Nelu Cazacu, Gabriela Tudor, "On microstrip parameter estimation", based on the design topology of the nonlinear transformation", 23rd International Symposium for Design and Technology in Electronic Packaging (SIITME), Constanta **– ISI Proceedings- IEEE**

It should be noted that papers 1, 2, 5, 6, 8 are indexed by ISI Proceedings and IEEE-Xplore.

Bibliography

[1] Marian Barbu, Sergiu Caraman, „Modelarea, Simularea si controlul bioproceselor”, Galati University Press, 2009, ISBN 978606-8008-41-7;

[2] R.P. Jones, “Measures of yeast death and deactivation and their meaning: Part I,” Proc. Biochem.,vol. 8, pp. 118-128, 1987;

[3] Rui D. Silva, Roberto Sotoca, Björn Johansson, Paula Ludovico, Filipe Sansonetty, Manuel T. Silva, José M. Peinado, Manuela Côrte-Real, "Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in Saccharomyces cerevisiae", September 2005;

[4] Peter Laun, Alena Pichova, Frank Madeo, Jörg Fuchs, Adolf Ellinger, Sepp Kohlwein, Ian Dawes, Kai-Uwe Fröhlich, Michael Breitenbach, "Aged mother cells of Saccharomyces cerevisiae show markers of oxidative stress and apoptosis", Molecular microbiology Journal,Volume 39, Issue 5 March 2001, Pages 1166–1173;

[5] Ning Wei, Axel Saalbach, Thorsten Twellmann, Tim W. Nattkemper, Erwin Flashel, “Identification of Cell Viability by an In-situ Dark-field Microscopic Sensor”, Electronic Journal of Biology, 2005, Vol. 1(2): 17-20;

[6] Olivier Frey, Fabian Rudolf, and Andreas Hierlemann, “HIGH-RESOLUTION LIVE CELL IMAGING OF THE YEAST LIFE CYCLE”, Bio Engineering Laboratory, Basel, Switzerland;

[7] M. Barbu, S. Caraman, G. Ifrim, G. Bahrim, E. Ceanga,“State Observers for Food Industry Wastewater Treatment Processes,” Journal of Environmental Protection and Ecology, vol. 12, pp. 678-687, 2011;

[8] Caraman, S., Ceangă, E., Frangu, L., Mencinicopschi, Gh., „Modelarea şi conducerea proceselor biotehnologice”, Editura Didactică şi Pedagogică, Bucureşti, pp. 263, 2002.

[9] Benavides, Micaela; Coutinho, Daniel; Hantson, Anne-Lise; et al. ”Robust Luenberger observers for microalgal cultures”, JOURNAL OF PROCESS CONTROL Volume: 36 Pages: 55-63 Published: DEC 2015.

[10] Amicarelli, Adriana; Quintero, Olga; di Sciascio, Fernando , “Behavior comparison for biomass observers in batch processes”, ASIA-PACIFIC JOURNAL OF CHEMICAL ENGINEERING Volume: 9 Issue: 1 Pages: 81-92 Published: JAN 2014.

[11] Coutinho, Daniel; Wouwer, Alain Vande , “A robust non-linear feedback control strategy for a class of bioprocesses”, IET CONTROL THEORY AND APPLICATIONS Volume: 7 Issue: 6 Pages: 829-841 Published: APR 2013

[12] Petre, Emil; Bunciu (Stanciu), Elena, “Adaptive and Robust-Adaptive Control Strategies for a Class of Fed-Batch Fermentation Processes”, CONTROL ENGINEERING AND APPLIED INFORMATICS Volume: 15 Issue: 1 Pages: 53-62 Published: MAR 2013

[13] Barrigon, Jose M.; Ramon, Ramon; Rocha, Isabel; et al., “State and specific growth estimation in heterologous protein production by Pichia pastoris”, AICHE JOURNAL Volume: 58 Issue: 10 Pages: 2966-2979 Published: OCT 2012

[14] Selisteanu, D.; Petre, E.; Roman, M.; et al., “Estimation of kinetic rates in a baker's yeast fed-batch bioprocess by using non-linear observers”, IET CONTROL THEORY AND APPLICATIONS Volume: 6 Issue: 2 Pages: 243-253 Published: JAN 2012

[15] Hulhoven, X.; Wouwer, A. Vande; Bogaerts, Ph., “State observer scheme for joint kinetic parameter and state estimation”, CHEMICAL ENGINEERING SCIENCE Volume: 63 Issue: 19 Pages: 4810-4819 Published: OCT 1 2008

[16] Hulhoven, X.; Wouwer, A. Vande; Bogaerts, Ph., “Hybrid extended Luenberger-asymptotic observer for bioprocess state estimation”, CHEMICAL ENGINEERING SCIENCE Volume: 61 Issue: 21 Pages: 7151-7160 Published: NOV 2006.

[17] Rapaport, A; Dochain, D., “Interval observers for biochemical processes with uncertain kinetics and inputs”, MATHEMATICAL BIOSCIENCES Volume: 193 Issue: 2 Pages: 235-253 Published: FEB 2005

[18] Goffaux, G; Vande Wouwer, A., “Bioprocess state estimation: Some classical and less classical approaches”, Edited by: Meurer, T; Graichen, K; Gilles, ED, Conference: Symposium on Nonlinear Control and Observer Design Location: Univ Stuttgart, Stuttgart, GERMANY Date: SEP 15-16, 2005

[19] “CONTROL AND OBSERVER DESIGN FOR NONLINEAR FINITE AND INFINITE DIMENSIONAL SYSTEMS”, Book Series: LECTURE NOTES IN CONTROL AND INFORMATION SCIENCES, Volume: 322 Pages: 111-128 Published: 2005

[20] Farza, M; Hammouri, H; Othman, S; et al., “Nonlinear observers for parameter estimation in bioprocesses” CHEMICAL ENGINEERING SCIENCE Volume: 52 Issue: 23 Pages: 4251-4267 Published: DEC 1997

[21] Caraman S., Barbu M., *Modelarea și conducerea proceselor biotehnologice. Lucrări practice. Volumul 1: Modelarea și estimarea stării și parametrilor proceselor biotehnologice*, Editura Fundației Universității ”Dunărea de Jos” din Galați, ISBN 973-627-240-0, 2005.

[22] http://www.apepur.ugal.ro/rezultate/RapStiintific_Observere_ro.pdf

[23] Bastin, G. şi Dochain, D., „On-line Estimation and Adaptive Control of Bioreactors”, Elsevier, Amsterdam, 1990.

[24] Selişteanu, D., „*Modelarea şi conducerea bioreactoarelor*”, Teză de doctorat, Universitatea din Craiova, 1999.

[25] Lewis, F., “Optimal Estimation with an Introduction to Stochastic Control Theory”, John Wiley & Sons, Inc.,1986

[26] Barbu, M., Caraman, S. Ceangă, E., „Stochastic Estimation Techniques for Biotechnological Processes”, In: Control Engineering and Applied Informatics, Vol. 6, No. 4, ISSN 1454-8658, pp. 43-51.,2004

[27] Barbu, M., Caraman, S. Ceangă, E., „State and Parameter Estimators for the Biosynthesis Processes”, 6th International Conference on Technical Informatics, CONTI’2004, Timisoara, Romania, Vol. 49, Pp. 139-144, Mai 27-28, 2004

[28] Valdinei Luís Belini, Philipp Wiedemann, Hajo Suhr, „In situ microscopy: A perspective for industrial bioethanol production monitoring”, Journal of Microbiological Methods 93 (2013) 224–232;

[29] Camisard V, Brienne J, Baussart H, Hammann J, Suhr H, „Inline characterization of cell concentration and cell volume în agitated bioreactors using în situ microscopy: application to volume variation induced by osmotic stress”, Biotechnol Bioeng. 2002 Apr 5;78(1):73-80;

[30] Tibayrenc P., Preziosi-Belloy L., Roger J.M., Ghommidh C., 2010, „Assessing yeast viability from cell size measurements”, J Biotechnol. 2010 Aug 20; 149(1-2): 74-80. doi: 10.1016/j.jbiotec.2010.06.019. Epub 2010 Jun 25;

[31] Christmann, Ingo Steinwart,”Support Vector Machines”, Book Information Science and Statistics 2008, ISBN: 978-0-387-77241-7 (Print) 978-0-387-77242-4;

[32] Ning Wei,1 Jia You,1 Karl Friehs,1 Erwin Flaschel,1 Tim Wilhelm Nattkemper2,” An In Situ Probe for On-Line Monitoring of Cell Density and Viability on the Basis of Dark Field Microscopy in Conjunction With Image Processing and Supervised Machine Learning”, Bielefeld University, Faculty of Technology, Applied Neuroinformatics

Group, Bielefeld,Germany-Published online 1 February 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21368;

[33] Rares Potcoava BUIGA," Raport stiintific - Proiect: 137/2014 (CTC-Videoscope)", Cod proiect: PN-II-PT-PCCA-2013-4-2289;

[34] Guillier L, Pardon P, Augustin J-C (2006), "Automated image analysis of bacterial colony growth as a tool to study individual lag time distributions of immobilized cells". J Microbiol Meth 65(2):324–334.

[35] Niblack W (1985), "An introduction to digital image processing". Strandberg Publishing Company, Birkeroed

[36] Pierre Tibayrenc, Laurence Preziosi-Belloy, Charles Ghommidh, „Single-cell analysis of S. cerevisiae growth recovery after a sublethal heat-stress applied during an alcoholic fermentation", J Ind Microbiol Biotechnol. 2011 Jun;38(6):687-96. doi: 10.1007/s10295-010-0814-6. Epub 2010 Aug 24;

[37] J.S. Guez, J.Ph. Cassar, F. Wartelle, P. Dhulster, H. Suhr, „The viability of animal cell cultures in bioreactors: Can it be estimated online by using in situ microscopy", Process Biochemistry Volume 45, Issue 2, February 2010, Pages 288–291;

[38] Shapiro, Linda and Stockman, George. "Computer Vision", Prentice-Hall, Inc. 2001

[39] Bittner C., Wehnert G. and Scheper T. (1998) In situ Microscopy for On-Line Determination of Biomass. Biotechnology and Bioengineering, 60(1): 24-35;

[40] Schilling J. (2004) Bioworld, 1: 8-9;

[41] Nattkemper T.W., Ritter H. and Schubert W. (2001) A Neural Classificator Enabling High-Throughput Topological Analysis of Lymphocytes in Tissue Sections. IEEE Trans. on Inf. Techn. In Biomed., 5(2): 138-149;

[42] Nattkemper T.W., Twellmann T., Schubert W. Et al. (2003) Human vs. Machine: Evaluation of Fluorescence Micrographs. Computers in Biology and Medicine, 33(1): 31-43.

[43] C. Ramya, V. Nirmala, "Cancer Detection by Cell Segmentation Using Clustering and Watershed Algorithms", International Journal of Innovative Research in Science, Engineering and Technology, volume 3, Special Issue 3, March 2014, ISSN (Online): 2319-875;

[44] L. Vincent and P. Soille, "Watersheds in digital spaces: An efficient algorithm based on immersion simulations," IEEE Trans. Pattern Anal.Machine Intell., vol. 13, pp. 583–598, 1991;

[45] Bleau and L. J. Leon, "Watershed-based segmentation and region merging," Computer Vis.Image Understand., vol. 77, no. 3, pp.317–370, 2000;

[46] J. M. Gauch,"Image segmentation and analysis via multiscale gradient watershed hierarchies", IEEE Trans. Image Process., vol. 8, pp. 69–79,1999;

[47] R.C.Gonzalez and R.E.Woods, Digital Image Processing.Englewood Cliffs, NJ, USA: Prentice-Hall, 2002;

[48] J. Park and J. M. Keller, "Snakes on the watershed," IEEE Trans. Pattern Anal. Mach. Intell., vol. 23, no. 10, pp. 1201–1205, 2001.

[49] S.-Y. Chen, S.-U. Chen, H.-Y.Wu, W.-J. Lee, Y.-H. Liao, and C.- K.Sun, "In vivo virtual biopsy of human skin by using noninvasive higher harmonic generation microscopy," IEEE J. Sel. Topics Quantum Electron., vol. 16, no. 3, pp. 478–492, 2010;

[50] H. Kobatake and S. Hashimoto,"Convergence index filter for vector fields", IEEE Trans. Image Process., vol. 8, no. 8, pp. 1029– 1038, 1999;

[51] Mehmet Sezgin and Bu lent Sankur „Survey over image thresholdingtechniques and quantitative performance evaluation", 13(1), 146–165 (January 2004);

[52] Pornphan Dulyakarn, Yuttapong Rangsanseri, and Punya Thitimajshima"Histogram transformation based threshold selection for Image Segmentation" 2000;

[53] https://en.wikipedia.org/wiki/Otsu%27s_method

[54] N. Bolshakova and F. Azuaje, "Clustering validation techniques for genome expression data," Genomic Signal Process., vol. 83, no. 4,pp. 825–833, 2003;

[55] E. Ceangă, I. Munteanu, A. Bratcu, M. Culea, Semnale, Circuite şi Sisteme, vol. 1: Analiza semnalelor, Ed. Academica, Galaţi, 2001, ISBN 973-8316-16-2

[56] L. Frangu, Recunoaşterea formelor şi prelucrarea imaginilor, Ed. Academica, Galaţi, 2001, ISBN 973-8316-18-9

[57] Jain, Anil K., Fundamentals of Digital Image Processing, Englewood Cliffs, NJ, Prentice Hall, 1989, pp. 150-153.

[58] Linda G. Shapiro and George C. Stockman (2001): "Computer Vision", pp 279-325, New Jersey, Prentice-Hall, ISBN 0-13-030796-3;

[59] Barghout, Lauren, and Lawrence W. Lee. "Perceptual information processing system." Paravue Inc. U.S. Patent Application 10/618,543, filed July 11, 2003;

[60] Batenburg, K J.; Sijbers, J.(2008) "Adaptive thresholding of tomograms by projection distance minimization". Pattern Recognition. 42 (10): 2297–2305;

[61] Batenburg, K J.; Sijbers, J. (June 2009). "Optimal Threshold Selection for Tomogram Segmentation by Projection Distance Minimization" .IEEE Transactions on Medical Imaging. 28 (5): 676–686;

[62] Kashanipour, A.; Milani, N; Kashanipour, A.; Eghrary, H. (May 2008)."Robust Color Classification Using Fuzzy Rule-Based Particle Swarm Optimization" . IEEE Congress on Image and Signal Processing.2: 110–114;

[63] Barghout, Lauren; Sheynin, Jacob (2013). "Real-world scene perception and perceptual organization: Lessons from Computer Vision". Journal of Vision. 13 (9): 709–709;

[64] R. Kimmel and A.M. Bruckstein.http://www.cs.technion.ac.il/~ron/PAPERS/ Paragios_chapter2003.pdf, International Journal of Computer Vision 2003; 53(3):225-243;

[65] R. Kimmel,http://www.cs.technion.ac.il/~ron/PAPERS/laplacian_ijcv2003.pdf, chapter in Geometric Level Set Methods in Imaging, Vision and Graphics, (S. Osher, N. Paragios, Eds.), Springer Verlag, 2003. ISBN 0387954880;

[66] Barghout, Lauren. Visual Taxometric approach Image Segmentation using Fuzzy-Spatial Taxon Cut Yields Contextually Relevant Regions. Communications in Computer and Information Science (CCIS). Springer-Verlag. 2014;

[67] Witold Pedrycz (Editor), Andrzej Skowron (Co-Editor), Vladik Kreinovich (Co-Editor). Handbook of Granular Computing. Wiley 2008;

[68] Barghout, Lauren (2014). Vision. Global Conceptual Context Changes Local Contrast Processing (Ph.D. Dissertation 2003). Updated to include Computer Vision Techniques. Scholars' Press. ISBN 978-3-639-70962-9;

[69] Barghout, Lauren, and Lawrence Lee. "Perceptual information processing system." Google Patents;

[70] Lindeberg, T.; Li, M.-X. (1997). "Segmentation and classification of edges using minimum description length approximation and complementary junction cues". Computer Vision and Image Understanding. 67 (1): 88–98;

[71] Ohlander, Ron; Price, Keith; Reddy, D. Raj (1978). "Picture Segmentation Using a Recursive Region Splitting Method". Computer Graphics and Image Processing. 8 (3): 313–333;

[72] Laurentiu Baicu, Sergiu Caraman, Laurentiu Frangu, Miron Mihaela, "Measurement of biomass concentration from a bioprocess by image processing techniques", ISEEE 2017 The 5th International Symposium on Electrical and Electronics Engineering, 20-22 October, Galati, Romania;

[73] X. Guo, F. Yu, "A Method of Automatic Cell Counting Based on Microscopic Image," 5thInternational Conference on Intelligent Human-Machine Systems and Cybernetics, Vol. 1, pp. 293-296, 2013;

[74] J.G.A. Barbedo, "A Review on Methods for Automatic Counting of Objects in Digital Images," Latin America Transactions, IEEE (Revista IEEE America Latina), vol. 10, pp. 2112-2124, 2012;

[75] T. Höpfner, A. Bluma, G. Rudolph, P. Lindner, T. Scheper, "A review ofnon-invasive optical based image analysis systems for continuous bioprocessmonitoring," Bioprocess Biosyst. Eng., vol. 33, pp. 247–256, 2010;

[76] Ceanga Emil, Nichita Cristian, Protin Ludovic, Cutululis Antonio, „Theorie de la commande des systemes", Ed. Tehnica, Bucuresti, 2001, pp. 370;

[77] Laurenţiu Baicu, Laurenţiu Frangu, George Ifrim, Sergiu Caraman, -- Control of the Yeast Growth Process Using an Image Processing-Based Transducer, 21st International Conference on System Theory, Control and Computing, October 19 - 21, 2017, Sinaia, Romania.-ISI Proceedings;

[78] Ranta, I., "On the Mathematical Modelling of Microbial Age Dynamic and Some Control Aspects of Microbial Growth Processes", Acta Polytechnica Scandinavica, Mathematics and Computer Science Series, nr. 35, Helsinki, 1982;

[79] J.M. Wildholm, "The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells," Stain Technol., vol. 47, pp. 189-194, 1972;

[80] C.J. Baker, M.M. Mock, "An improved method for monitoring cell death in cell suspension and leaf disc assays using Evans blue," Plant Cell Tissue Organ Cult.,vol. 39, pp. 7-12, 1994;

[81] L.A. Castro-Concha, R.M. Escobedo, M. de Lourdes Miranda-Ham, "Measurement of Cell Viability," in. V.M. Loyola-Vargas, N. Ochoa-Alejo (eds.), "Plant Cell Culture Protocols," Methods in Molecular Biology, Humana Press, vol. 877, pp. 49-56, 2012;

[82] M. Bouix, J.-Y. Leveau, "Rapid Assessment of Yeast Viability and YeastVitality During Alcoholic Fermentation," Journal of the Institute of Brewing, vol. 107, pp. 217-225, 2001;

[83] A.R. Boyd, T.S. Gunasekera, P.V. Attfield, K. Simic, S.F. Vincent, D.A. Veal, "A flow-cytometric method for determination of yeast viability and cell number in a brewery," FEMS Yeast Research, vol. 3, pp. 11-16, 2003;

[84] N. Wei, J. You, K. Friehs, E. Flaschel, T.W. Nattkemper, "An In Situ Probe for On-Line Monitoring of Cell Density and Viability on the Basis of Dark Field Microscopy in Conjunction With Image Processing and Supervised Machine Learning," Biotechnol. Bioeng., vol. 97, pp. 1489-1500, 2007;

[85] N. Wei, A. Saalbach, T. Twellmann, T.W. Nattkemper, E. Flashel, "Identification of Cell Viability by an In-situ Dark-field Microscopic Sensor," Electronic Journal of Biology, vol. 1, pp. 17-20, 2005;

[86] N. Wei, J. You, K. Friehs, E. Flaschel, T.W. Nattkemper, "In situ dark field microscopy for on-line monitoring of yeastcultures," Biotechnol. Lett., vol.29, pp. 373-378, 2007;

[87] S. Burgemeister, T.W. Nattkemper, T. Noll, R. Hoffrogge, E. Flaschel, “CellViCAM—Cell viability classification for animal cellcultures using dark field micrographs,” Journal of Biotechnology, vol. 149, pp. 310-316, 2010;

[88] P. Wiedemann, M. Worf, H.B. Wiegemann, F. Egner, C. Schwiebert, J. Wilkesman,J.S. Guez, J.C. Quintana, D. Assanza, H. Suhr, “On-line and real time cellcounting and viability determination for animal cell process monitoringby in situ microscopy,” BMC Proceedings,vol. 5(Suppl 8), p. 77, 2011;

[89] Laurențiu Marius Baicu, George Ifrim, Laurențiu Frangu, Sergiu Caraman, “”Viability diagnosis in biotechnological cultures through image processing, 19th International Conference on System Theory, Control and Computing, oct. 2015, Cheile Gradistei, Romania, pp 770-775; - ISI Proceedings

[90] http://www.biocon.ugal.ro/

[91] Tofan C., Bahrim G., Nicolau A., Zara Margareta, Microbiologia produselor alimentare – Tehnici şi analize de laborator, Editura Agir, ISBN 973-8130-89, 2002;

[92] Laurențiu Baicu, George Ifrim, Vasilica Barbu, Laurențiu Frangu, Sergiu Caraman, “Stage evaluation of cell growth in yeast culture through image processing”, 20th International Conference on System Theory, Control and Computing, October 2016, Sinaia, Romania, pp.704-709; - ISI Proceedings