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Chapter 8. AUTOMATIC ALGORITHM TO MEASURE PHB ACCUMULATION AND SIMILAR STRUCTURES

8.1. Introduction

Along with the rapid technological progress, social awareness and demand for innovative materials, there is a growing need for environmentally friendly alternative to conventional plastics. One of the promising biopolymers is PHA (polyhydroxyalkanoate) and its derivatives, which depending on the hydroxy acid length, can create materials with extremely different properties, from hard and brittle to elastic and sticky. The main advantage is the ability to regulate their features, such as their resistance to mechanical factors or degradation rate, by introducing modifications in the monomer ratio or by combining them with other materials [1]. An additional superiority in ecology terms is the possibility of using waste materials as a substrate. As a result, modern biopolymers are more often used in numerous industry and medicine sectors. The high biocompatibility of PHB (polyhydroxybutyrate) contributes to the great interest in its use for the controlled drug release into the patient's body. The most important aspect in this matter is that the foreign body insertion made of the above-mentioned biopolymer does not cause strong defense reactions in the organism, and its response is mild [2]. Currently, PHA derivatives are also used in the treatment of bone defects and cartilage tissues, cardiovascular engineering [1], as well as in dressing material production and fine powder as a lubricant for surgical gloves [2]. The biocompatible surfactant allowed to

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use the hydrophobic PHB fiber to produce a material that can replace cotton swabs during surgical procedures. This invention eliminates the risk of complications resulting from leaving a fiber fragment inside a patient, as it degrades without accompanying reactions that could be dangerous for the human body. The list of biomaterial applications is growing every year, leading to the emergence of new solutions in terms of production and processing. The process of obtaining biopolymers as a microbial metabolism product also involves quantitative control and microscopic observations to confirm their efficiency. Due to the lack of a tool that may greatly facilitate and control the PHB production process, there is a need to create an algorithm that provides information that allows for the assessment of the material production efficiency.

8.2. Materials and methods

8.2.1. Culture of microorganisms

The culture of *Pseudomonas fluorescens*, capable of biopolymer accumulation under certain conditions, was maintained in two bioreactors operated respectively in a batch and continuous modes. The research was aimed at optimizing the PHB production process, which is one of the intracellular metabolites. To increase the fatty acid conversion into a PHA derivate, it was necessary to maintain stressful conditions for microorganisms and this could be achieved by preventing the microorganisms from gaining access to a carbon source. In the first bioreactor, a fed-batch culture was carried out, involving cyclic metabolite exchange and nutrient additions. For this purpose, each day an amount of reactor medium was decanted and replaced with a freshwater containing the nutrient. The supernatant obtained was taken for further analysis. Homogenous concentrations of oxygen and nutrient were maintained in the entire vessel by using mechanical stirrer and aeration system. Continuous culture experiments were performed in a 5-liter Sartorius Biostat A + bioreactor, providing optimal conditions for *Pseudomonas fluorescens*. The optimal temperature 30°C was controlled by using a heating blanket and deviations from the optimal pH = 7 were adjusted by adding a buffer solution ($NaHCO_3$) to the vessel. Continuous control of breeding parameters was possible due to computer monitoring equipment, which included level sensors, dissolved oxygen, pH, and temperature sensors.

In both bioreactors, the stressful conditions necessary for the production and subsequent accumulation of PHB were maintained by using a medium consisting of peptone and olive oil in varying proportions. The peptone was the main carbon source, while the fatty acid esters contained in the oil were crucial in terms of cell stress, and their presence was evidenced by the increase in lipase activity. Periodic measurements of ammoniacal nitrogen, chemical oxygen demand, lipase activity, and biomass concentration were performed to determine favorable conditions for the target process. The culture condition was controlled on the basis of the concentration of ammoniacal nitrogen. A progressive extinction of microorganisms was manifested by its low value and low nutrient degradation. The degree of nutrient consumption was determined based on lipase activity, as this enzyme is responsible for fat digestion. Measurements on a spectrophotometer were made on a Secoman Uvi Light XT 5 using a p-Nitrophenol. The procedure assumed recording the values at intervals of 15, 25, and 35 minutes from the start and the final result in the form of a waveform for 410 nm and 290 nm. The high lipase activity observed in connection with the determination of the high COD result was interpreted as the significant amount of organic substances and the presence of residual oil in the reactor.

8.2.2. Acquisition of microscopic images

In order to confirm the PHB accumulation in the activated sludge a series of microscopic observations were made. A standard fixation method in the flame was used to prepare the collected sample, then the sample was stained by using two developed methods. The first of staining method uses Sudan III dye and is aimed at the identification of lipids, triglycerides, and lipoproteins. The fixed plates are placed in a humidity chamber, thoroughly rinsed with dye, and left for 30 minutes. The Sudan III (0.1% dye solution in 90% ethyl alcohol solution) is a fat-soluble, nonpolar substance that interacts hydrophobically with hydrocarbon lipid chains, dyeing them red. The second staining procedure was used to observe the PHB granules that are stained in a light orange color. The staining reaction takes place due to the water--soluble substance Nile Blue A (0.05 g of Nile Blue sulfate, 100 ml of 96% ethanol), which is a fluorescent dye from the oxazine group. The procedure involves incubating the samples for 10 minutes in a Coplin staining jar placed at 50°C. Both procedures were terminated by carefully washing the excess dye from the plates and allowing them to dry. For the continuous control of the PHB accumulation process, samples were prepared each day, followed by observations under fluorescence and confocal microscopes. The FV1000 microscope from the Olympus company was used for PHB observations. Figure 1 is an exemplary image obtained during the observation of a specimen, stained with Nile Blue A.

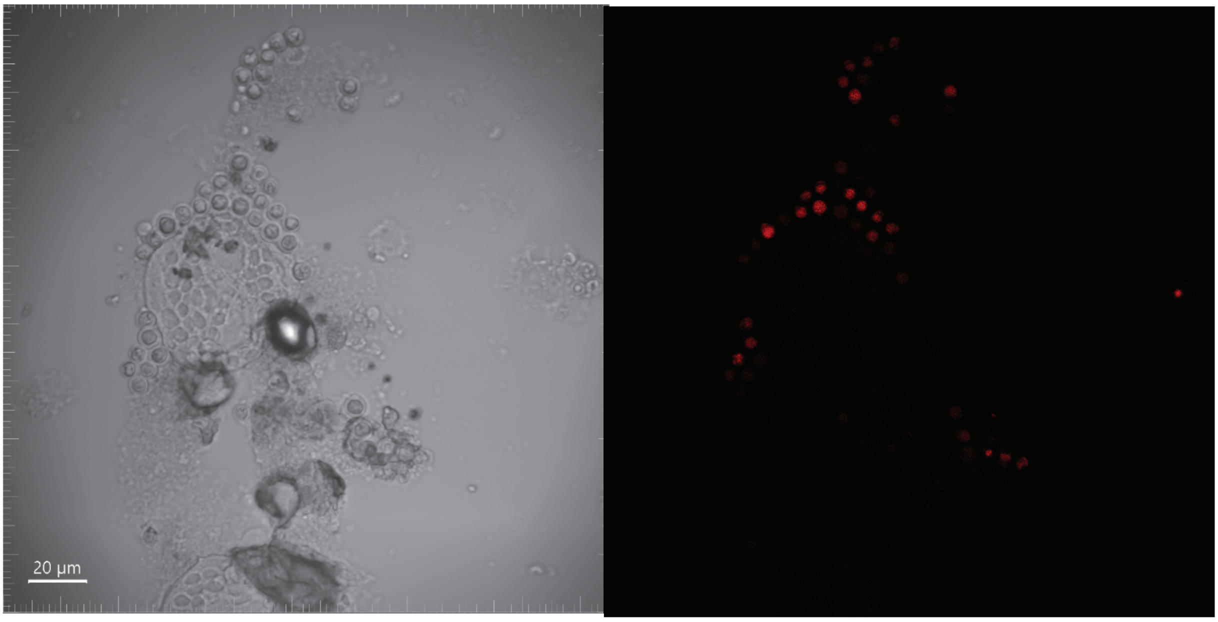

Fig. 1. Comparison of the microscopic images in visible light, and after using a red filter, and excitation with a blue laser. Nile blue A – stained sample was observed using a 60x objective

Rys. 1. Porównanie obrazu próbki mikroskopowej w świetle widzialnym i po zastosowaniu filtra czerwonego oraz wzbudzeniu laserem niebieskim. Próbkę wybarwioną błękitem Nilu A obserwowano przy użyciu obiektywu 60x

8.2.3. Tools and methods used to create the algorithm

The algorithm determining the percentage of PHB in the observed area was written in Matlab programming language, which provides wide packages range for digital image processing. The fact that PHB in the photos is present in the form of the regular objects, similar in shape and color was used. The appropriate RGB channel binarization and morphological operations and filters were applied to identify the background, floc, and granules. To prevent dye misidentification as PHB not rinsed out, the circularity factor was calculated. Too divergent results were treated as misdiagnoses. The main algorithm result is the polymer percentage calculated in the floc and the processed image with identified object's contours marked on it. To visualize the size distribution of the PHB granules, granulometry analysis was performed. Finally, the program returns two charts. The first one is a histogram that shows the area relation of the identified objects, their number with the marked mean, standard deviation, and median. It also presents the surface area distribution. Then, the boxplot displays additional information about outliers. The operation of the algorithm is shown in Fig. 2.

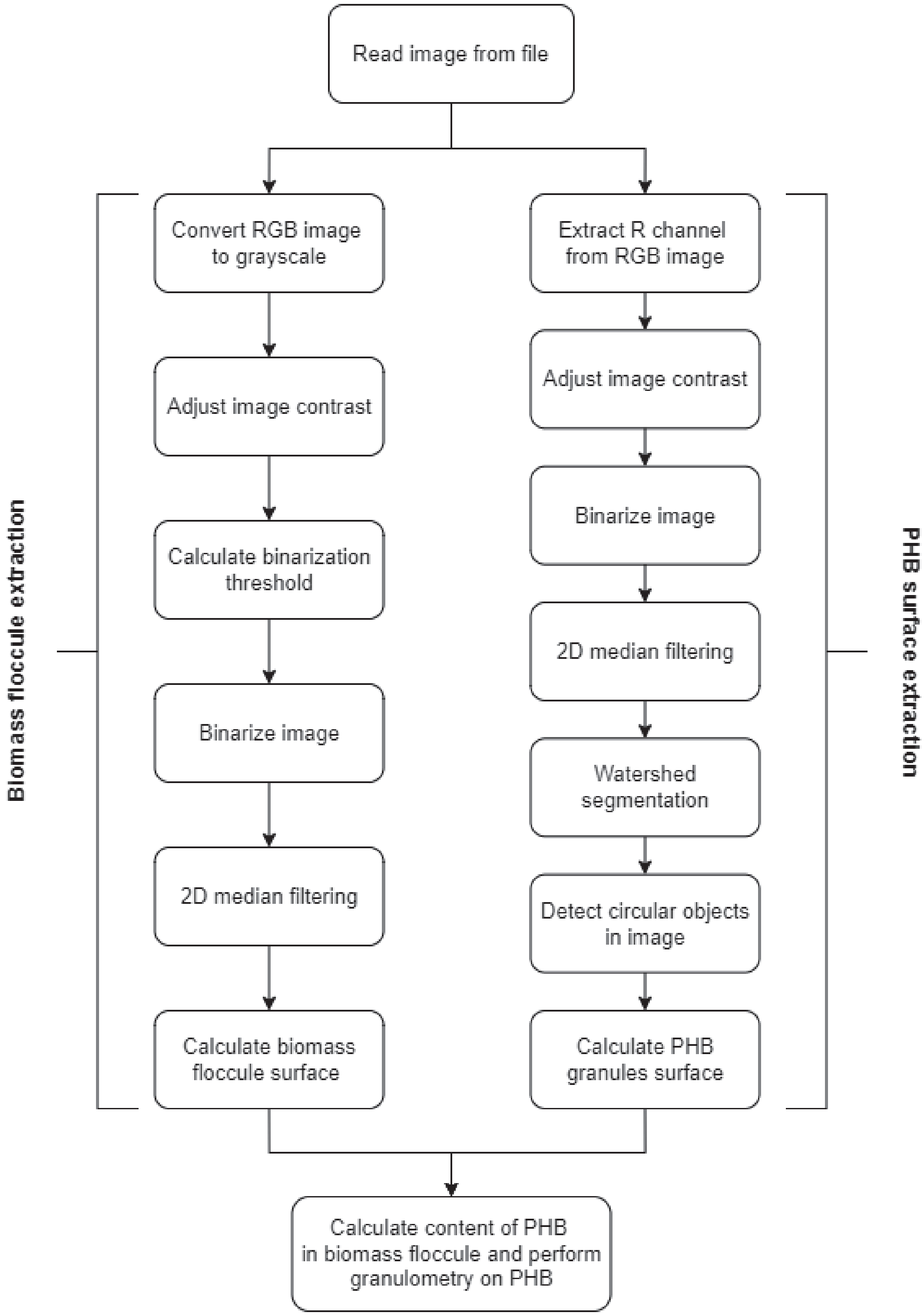

Fig. 2. Operating scheme of the algorithm
Rys. 2. Schemat działania algorytmu

8.3. Results and discussion

The confocal microscopy allows for image generation in three channels, spatial PHB granules observation, and other structures. The proposed algorithm is applied to analyze the microscopic image obtained. In this case, it was used to confirm the presence of PHB granule in the stained samples. The microscopic images show clusters of regular objects with high fluorescence, identified as biomaterial granules. When there are characteristic areas in the image, the algorithm returns a histogram describing the PHB amount over the entire surface, an image with detected, selected objects, and a box plot showing the expected value and standard deviation. In this case, for this image, changing the bins on the histogram will not make the distribution closer to the normal distribution. The program allows for determining the percentage of polymer granules in the identified structure. The algorithm improves the subsequent stages of planning experiments. The result of the microscopic image analysis is shown in Fig. 3. This is a simple way to confirm the accumulation of PHB and estimate its amount in bacterial cells.

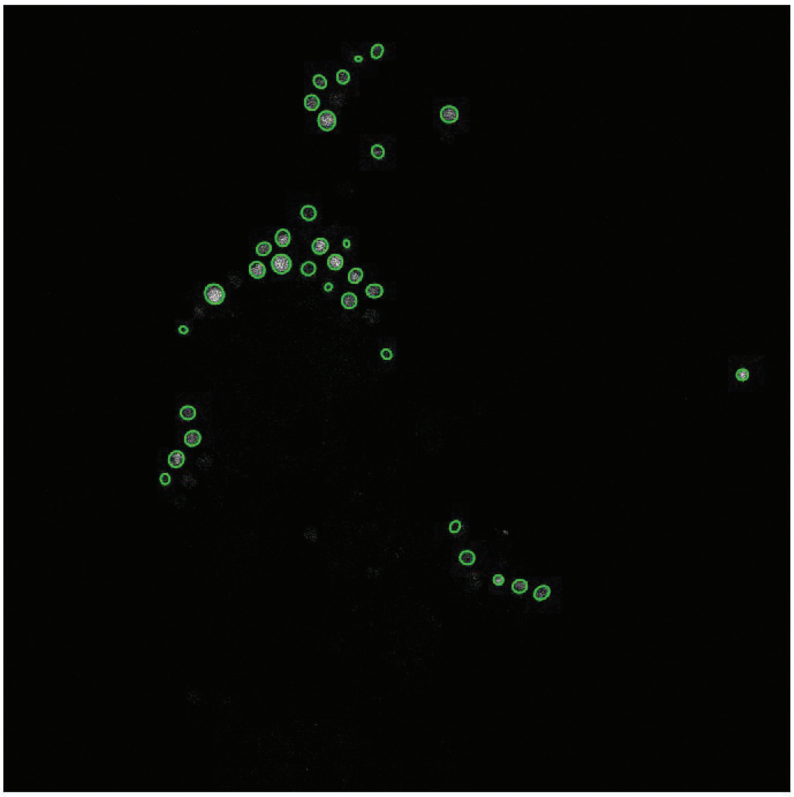

Fig. 3. The microscopic image processed by the proposed algorithm (Fig. 1)
Rys. 3. Rezultat działania algorytmu na obrazie mikroskopowym (Rys. 1)

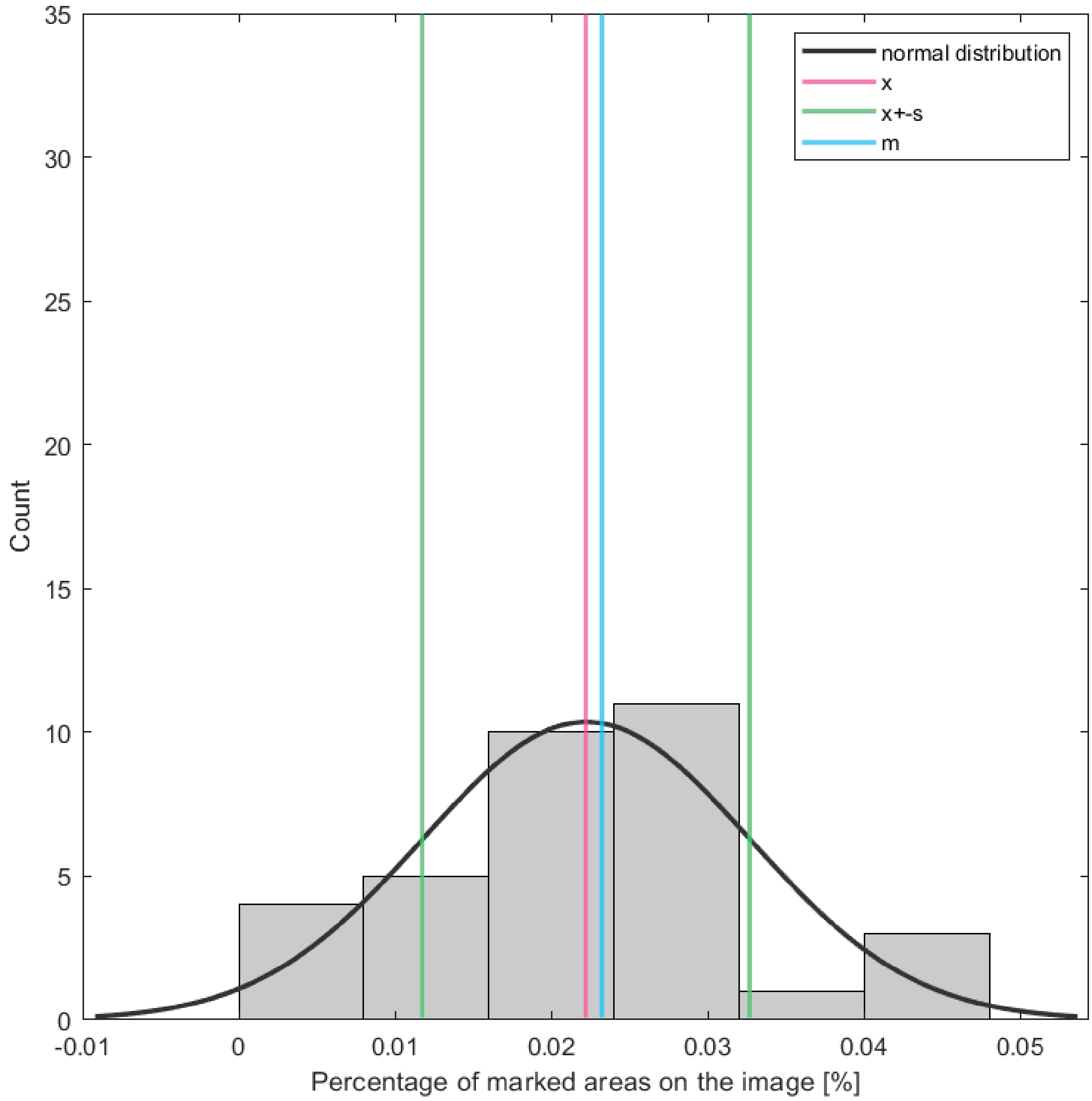

Fig. 4. Histogram showing the percentage of polyhydroxybutyrate in the microscopic image (Fig. 1) and marked with vertical lines: m – median, x – mean, ± s – standard deviation

Rys. 4. Histogram przedstawiający procent polihydroksymaślanu na obrazie mikroskopowym (Rys. 1), zaznaczono liniami pionowymi: m – medianę, x – średnią, ± s – odchylenie standardowe

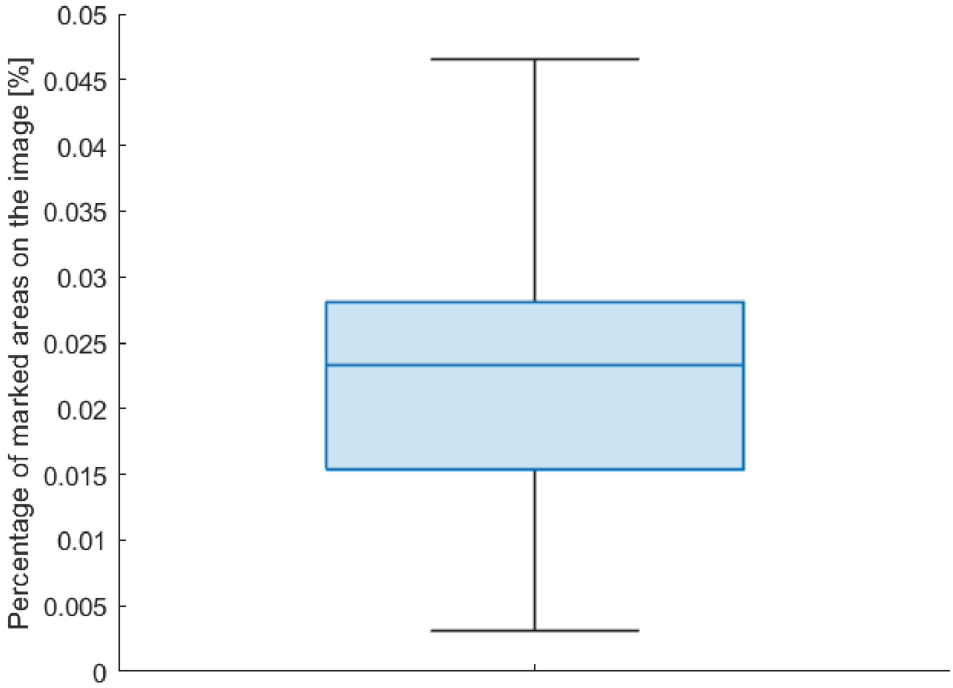

Fig. 5. Box plot showing the percentage of identified polyhydroxybutyrate in the microscopic image (Fig. 1)

Rys. 5. Wykres pudełkowy zawierający procent polihydroksymaślanu na obrazie mikroskopowym (Rys. 1)

The algorithm is universal in a certain way. It can also be used to detect and perform the described operations on objects similar to PHB granules. An example of its application can be the results of an experiment with HELA cells. Carefully prepared HELA tumor cells with fluorescently labeled histone H2B were introduced into the system to assess their cell cycle length and to record monitoring of the movements of individual daughter cells. The research sample was treated with the drug doxorubicin. The experiment was made observations on a fluorescence microscope with automatic image acquisition software. Figure 6 – Fig. 9 present the operations performed on the above-mentioned example to identify cells and their percentage in the photos.

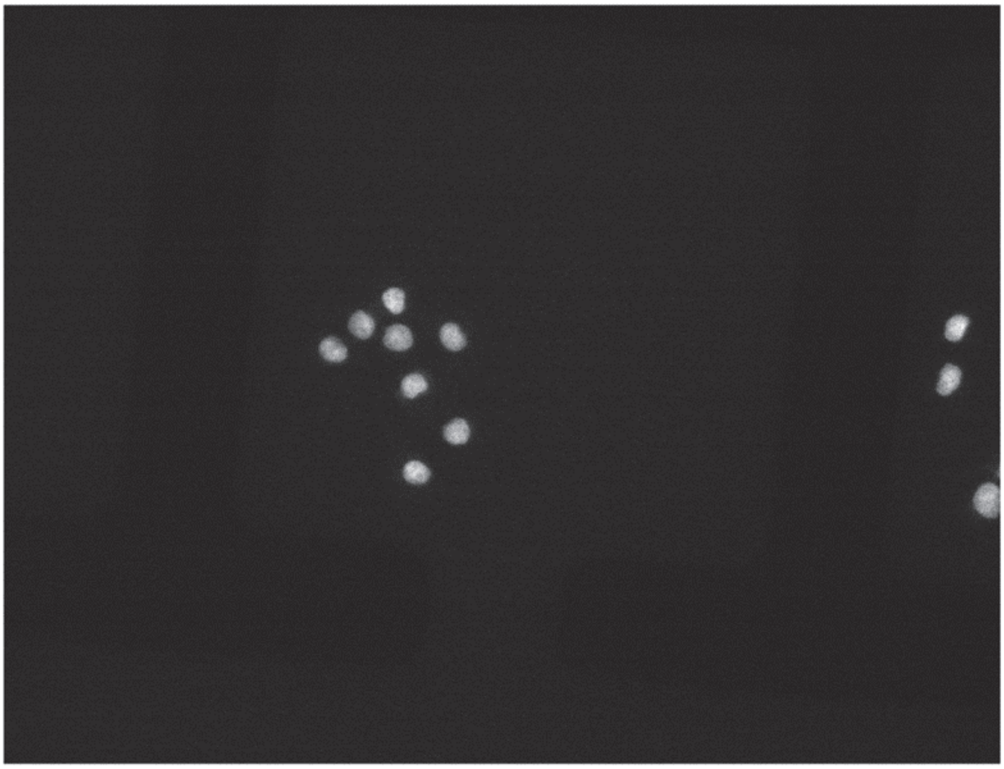

Fig. 6. Sample microscope image with HeLa cells
Rys. 6. Przykładowy obraz mikroskopowy z komórkami HeLa

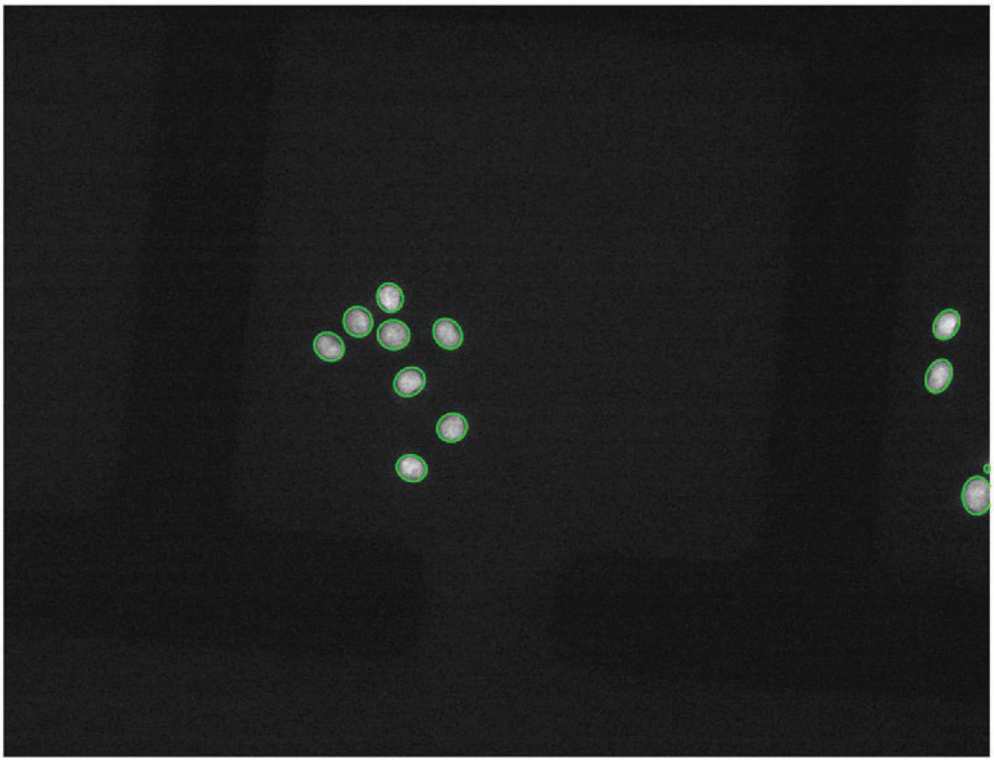

Fig. 7. The processed image after applying the proposed algorithm for the microscopic image (Fig. 6)
Rys. 7. Wynik działania algorytmu na obrazie mikroskopowym (Rys. 6)

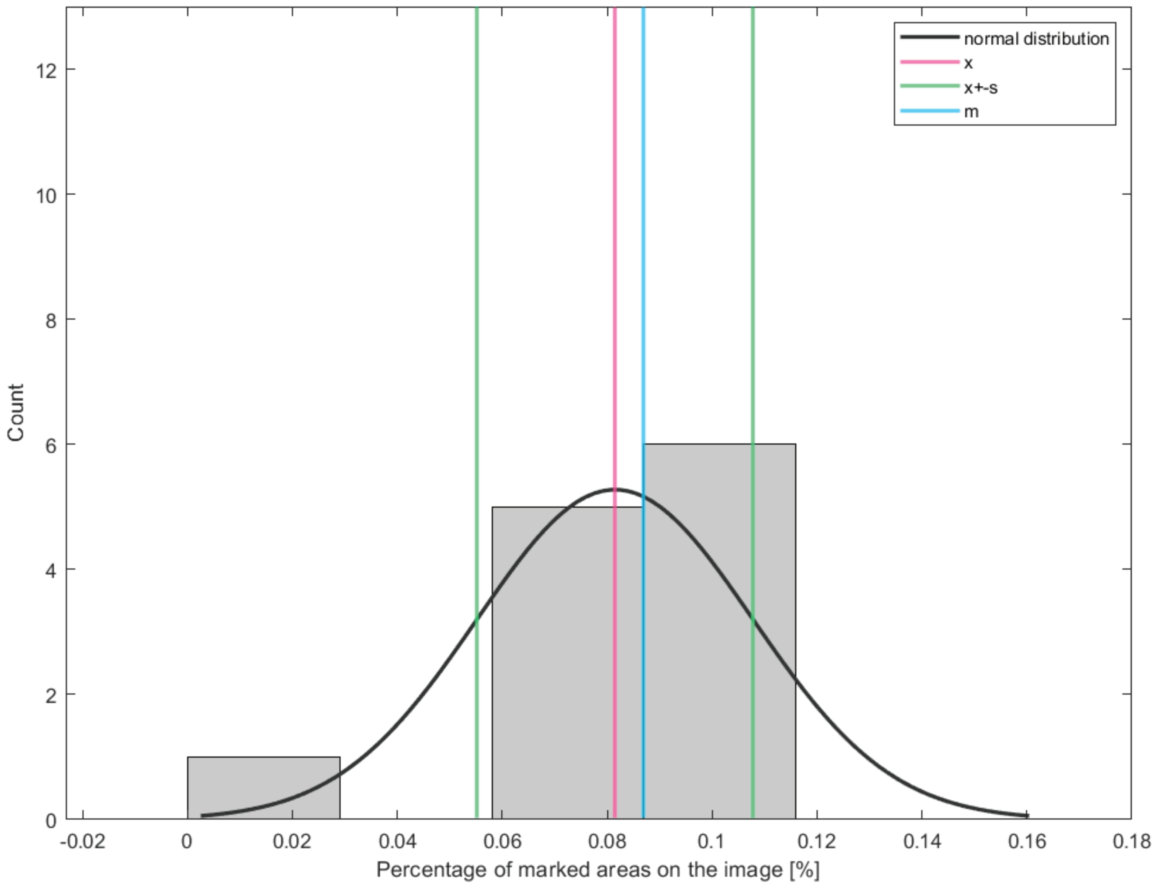

Fig. 8. Histogram showing the percentage of marked areas on the microscopic image (Fig. 6) and marked with vertical lines: m – median, x – mean, ± s – standard deviation

Rys. 8. Histogram przedstawiający procent zaznaczonych obszarów na obrazie mikroskopowym (Rys. 6), zaznaczono liniami pionowymi: m – medianę, x – średnią, ± s – odchylenie standardowe

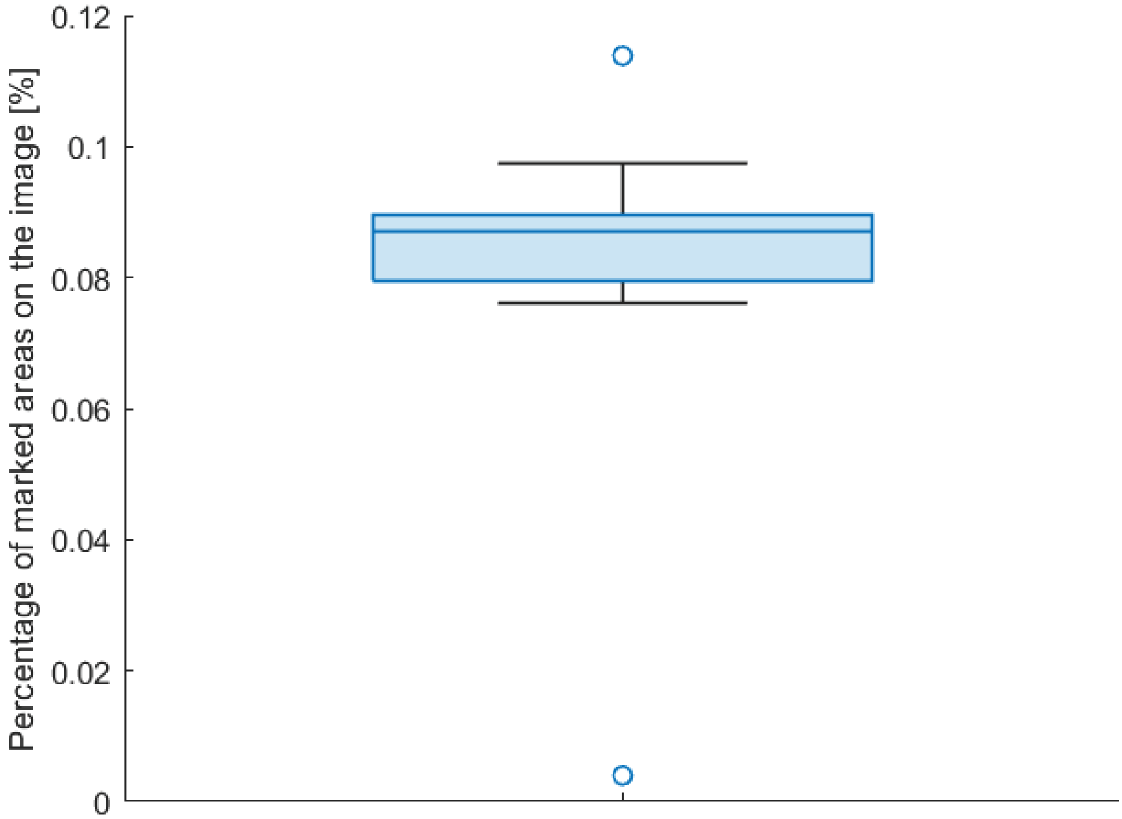

Fig. 9. Box plot showing the percentage of marked areas in the microscopic image (Fig. 6)
Rys. 9. Wykres pudełkowy zawierający procent zaznaczonych obszarów na obrazie mikroskopowym (Rys. 6)

Biomaterials are a promising alternative to plastics used so far, both in application versatility, waste management, and minimizing their generation terms. Some by-products from industrial processes can be used to produce PHA, thus it is possible to reduce problem needed to produce PHA processing costs by up to about 50%. One of the promising alternatives to an expensive substrate are waste substances from the oil industry, which are classified as attractive carbon sources for microorganisms. Another potential suppliers are agriculture and the food industry. By-products or materials rejected during individual processes can be reused in environmentally friendly biopolymer fabrication while reducing their production costs. Potentially suitable for ecological alternative production are banana parts and the peel of numerous fruits, sugar cane fragments discarded in various processing steps, and algae biomass [3]. In the research, olive oil was used for this purpose but leftover fry oil can also be used. Optimizing the PHB production process by reducing the raw materials and energy consumption while maximizing the biosynthesis efficiency requires continuous observations and polymer accumulation control. The proposed algorithm makes it possible to facilitate and automate these tasks, and to estimate the percentage of granules in microscopic images. On this basis, the solutions legitimacy, the microorganism breeding system, or the supplying substrates method was determined. Visualizing the results in graph form showing the object number with size within a given range facilitates work and helps in determining the systematization of the characteristics of the granules. The need to conduct long-term observations generates large amounts of data for analysis. Using a program that performs some of the tasks not only relieves the research team but often enables control activities to be carried out in time, allowing for a quick response to changes observed in the results. In addition, the universality of the algorithm allows it to be used in subsequent studies. The biomaterials topic, and especially PHA and its derivatives, has not been sufficient, and the work continuation may bring huge profits to medicine. Biopolymers have numerous applications in many areas, such as bone and cartilage tissue engineering. One in vivo study on the bone treatment defects has shown that PHB can be applied in implant production and other bone marrow scaffolds. It was a positive tissue adaptive response to the polymer. In addition, a 12-month follow-up showed no chronic inflammatory response. Other applications of PHA derivatives are, for example, devices for the repair of meniscus, articular cartilage, or tendons, as well as bone plates, bone plating systems, orthopedic pins, ligament and tendon grafts, and bone graft substitutes [1]. The antibacterial properties of some biomaterials application in the prophylaxis and postoperative infection treatment have also been shown. For this purpose, a connecting PHB spheres method containing antibiotics with poly(ethylene glycol) (PEG) and titanium, which can be the base material for implants, was developed. Titanium surface with antimicrobial properties in surgical procedures reduces bacteria adhesion and eliminates some of the infection risks [5]. Another department that eagerly uses biopolymer solutions in cardiovascular tissue engineering, deals with a health problem group that is at the forefront in incidence terms. Unfortunately, many cases of cardiovascular disease require surgical treatment, part of which is the patch applied to the patient's body. The material from which the patch is made must meet several requirements, including not being toxic or immunogenic, and also characterized by high durability and flexibility [1]. PHB is a polyester whose thermal and mechanical properties can be compared with conventional plastics [4]. Again, biopolymers can meet the requirements and their application can greatly improve treatment methods and overcome the problems associated with conventional solutions. One of them is the need to repeat the heart valve replacement procedure because if the patient was a child at the first operation time, the implanted implant becomes a mismatch with the organ after some time. By using the PHB to impregnate the valve, an increase in its volume was observed in vivo. In this way, the idea of an implant that adapts to the recipient's body was born, which would exclude the additional risk of postoperative complications. Biopolymers are commonly used mainly in surgery, but not only. In addition to the examples described above, the biopolymers are also useful in the production of sutures and suture fasteners, staples, rivets, screws, surgical meshes, fillers, repair patches, adhesion barriers, guided tissue repair or regeneration devices, nerve guides, pericardial patches, vein valves, ocular cell implants, spinal fusion cages, skin substitutes, and many more [1]. Due to the strength and properties similar to conventional plastics, some biopolyesters can also be a material for film production that is a part of food packaging and the production of disposable cutlery [4]. The wide range of possibilities offered by PHA application derivatives only confirms the belief that biopolymers will be one of the key materials in the field of medicine, health protection and environmental engineering. Thus, optimizing their production process is inevitable. Thanks to the proposed algorithm, it is possible to facilitate the production process and automate some tasks.

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Abstract

Over the years, biomaterials have become more and more popular and find a new applications huge number. This is due to the growing social awareness and the need to implement ecological solutions. Environmentally-friendly production and the possibility of using industrial waste are just some of the biopolymer advantages. A wide property range and modification potential allow them to be used in many industry and medicine sectors. Biomaterials are applied both in surgical tools and accessories production as well as implants permanently inserted into the patient's body. Features such as biocompatibility and high durability eliminate the problems associated with conventional solutions. One of the most widely used biomaterials is PHA derivatives, which can be obtained as a metabolic product of *Pseudomonas fluorescens* bacteria. Process optimization requires the maintenance methods and control procedures necessary for breeding development to determine its effectiveness. One way to confirm the accumulation of PHB is by microscopic observation. An algorithm was developed that automated the identifying polymer granule step. It also improved the process of estimating the PHB amount in an image and allowed for convenient results visualization. The script is also used in other studies where data of this type is analyzed.

Keywords: polyhydroxybutyrate (PHB), polyhydroxyalkanoates (PHA), bioplastic, PHB accumulation, measure PHB accumulation