Journal of Computer Science and Technology Studies
ISSN: 2709-104X
DOI: 10.32996/jcsts
Journal Homepage: www.al-kindipublisher.com/index.php/jcsts

JCSTS
AL-KINDI CENTER FOR RESEARCH AND DEVELOPMENT

| RESEARCH ARTICLE

Quantitative Analysis and Automatic Counting of Microorganisms Using Image Processing Techniques

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| ABSTRACT
Microorganisms play critical roles in many fields, including medicine, food, and industrial production. Although the quantitative analysis of these organisms is a fundamental process for scientific research, manual counting through visual observation is a highly time-consuming and subjective process for experts. In this study, an automated counting system based on digital image processing techniques was developed to address the disadvantages of manual counting methods. The study utilized 170 microscopic images obtained from 22 different microfungus species. Two distinct methods based on the Circular Hough Transform (CHT) and morphological operations were tested. The results demonstrated that the CHT method, with a precision value of 0.5, exhibited a high correlation of 0.98 with expert counting.

| KEYWORDS
Image Processing, Microorganism, Microfungus, Hough Transform, Automatic Counting.

| ARTICLE INFORMATION
ACCEPTED: 10 April 2026 **PUBLISHED:** 17 May 2026 **DOI:** 10.32996/jcsts.2026.8.7.6

1. Introduction

Microorganisms are very small organisms that cannot be seen with the naked eye but can be observed under a light or electron microscope (Madigan et al., 1997). Classification standards vary, and there are many different types of microorganisms. Microorganisms generally consist of bacteria, microfungi, and viruses. Determining the quantity of such organisms as bacteria, microfungi, and viruses is of vital importance in food safety, disease diagnosis, and environmental monitoring processes. Some microorganisms endanger human health by causing food spoilage, infecting people, and causing diseases, but some microorganisms are also beneficial to humans. Penicillin is a life-saving and very important discovery in the field of medicine. Nevertheless, yeast is used in industrial fermentation, ethanol production, and food production for humans (Brill, 1981). Additionally, many microorganisms found in the intestines of healthy people can help humans break down and absorb food and toxic substances. Industrial production and the human body can be harmed by certain microorganisms (Zhang et al., 2021).
Microorganisms play a crucial role in human daily life. In this context, while beneficial microorganisms are utilized in daily life, harmful microorganisms must be prevented (Zhang et al., 2021). Microbial counting is widely used in safety testing in the food and pharmaceutical industries, in biomedical testing, and in environmental monitoring (Liu et al., 2004). Currently, there are two main methods for microbial counting and measurement: manual counting by an expert and counting using computer image analysis (Rajapaksha et al., 2019). Manual counting performed by an expert primarily uses plate counting, hemocytometry, and turbidimetry. In the plate counting method, bacteria are placed on a suitable culture medium and then allowed to grow into colonies. Once sufficient growth is observed, an expert determines the number of colonies under a microscope. An advantage of the plate count method is that it estimates the number of live bacteria. However, the process is somewhat labor-intensive, and culturing microorganisms and obtaining results takes time. The actual number of live bacteria is lower than the number of

colonies obtained, because two or more live bacterial cells are defined as a colony when they adhere to one another (Balestra and Misaghi, 1997).

1.1 Microfungi

A group of eukaryotic microorganisms, including molds, yeasts, and fungi, which can produce spores through asexual and sexual reproduction, are known as microfungi. Tinea pedis is a widespread foot skin disease worldwide. There are no sebaceous glands on the soles of the feet or between the toes; therefore, filamentous fungi can thrive in an environment lacking fatty acids and with reduced air circulation (Perea et al., 2000). Fungi are known as heterotrophs and obtain their nutrients either by causing the decomposition of dead organic matter or by parasitizing a living host. Asexual spore formation is a more common method of reproduction. These spores are approximately 5 µm–15 µm in size and can spread over large areas. They can contaminate many things, including food, and can cause diseases in plants and animals, as well as act as allergens for humans. Unlike bacteria, the cell walls of fungi consist of fibrous structures. The lifespan, shape, size, and color of fungi can vary greatly (Bıyık and Asan, 2022). Among airborne allergens and fungal spores that trigger various diseases, Aspergillus sp. spores were the first to be identified. Spores of Aspergillus spp., Penicillium spp., Cladosporium spp., and Alternaria spp. are common fungal allergens in our country (İmalı et al., 2011a; İmalı et al., 2011b; Sugeçti et al., 2018).

2. Materials

From the collection housed in the University-Industry-Public Cooperation Development Application and Research Center (ÜSKİM) laboratories at Kahramanmaraş Sütçü İmam University, 22 microfungi were selected for use in this thesis study and inoculated by an expert onto media appropriate for their respective species, as shown in Table 1. The characteristics of Penicillium, Aspergillus, and Cladosporium species were taken into account when selecting the microorganisms.

Table 1. Number of images obtained from the light microscope (Bağlar, 2023)

PREPARATION IDENTIFICATION				
No.	**CULTURE MEDIUM**	**DATE**	**SAMPLE NAME**	**Number of Images (Count)**
1	PDA	April 7, 2021	Penicillium sp	2
2	PDA	April 7, 2021	Penicillium sp	10
3	PDA	April 7, 2021	Penicillium sp	10
4	PDA	April 7, 2021	Aspergillus niger	10
5	PDA	April 7, 2021	Penicillium sp.	10
6	PDA	April 7, 2021	Cladosporium sp	10
7	PDA	April 7, 2021	Cladosporium sp	10
8	PDA	April 7, 2021	Penicillium sp.	10
9	PDA	April 7, 2021	Aspergillus sp	10
10	PDA	April 7, 2021	Aspergillus sp	10
11	PDA	April 7, 2021	Penicillium sp.	10
12	PDA	April 7, 2021	Penicillium sp.	10
13	MHA	April 8, 2021	Penicillium sp.	10
14	MHA	April 8, 2021	Penicillium sp.	7
15	PDA	April 8, 2021	Penicillium sp.	7
16	PDA	April 8, 2021	Aspergillus sp.	7
17	MHA	April 8, 2021	Penicillium sp	1
18	PDA	April 8, 2021	Cladosporium sp.	9
19	SDA	April 8, 2021	Penicillium sp	6
20	PDA	April 8, 2021	Aspergillus sp.	4
21	SDA	April 8, 2021	Penicillium sp	3

22	PDA	April 8, 2021	Cladosporium sp.	4

2.1 Preparation of Specimens for the Dataset

In this study, some microfungal samples were examined as references. The preferred solution for the microscopic examination of microfungal species is cotton blue lactophenol. We prepared a 30-gram solution dissolved in 10 mL of distilled water. The ratios of lactic acid, phenol, and glycerin are 1:1:1. We carefully drop the prepared solution onto a slide, then use a sterile loop to remove the fruiting bodies and hyphae of the microfungi, and cover the structure with a coverslip. As shown in Figure 1, all edges of the prepared slides are sealed and isolated with nail polish (Hasenekoğlu, 1990; Koçer, 2012).

Figure 1. Prepared sample slide

2.2 Image Acquisition Process from Preparations for the Dataset

To create the dataset, 170 images were obtained from each microorganism in the transparent specimens by selecting appropriate lighting and a 40X magnification ratio. This process was performed using the Nikon ECLIPSE 80i illuminated microscope, which comes with the Nikon DS-Ri1 camera, and a 4 GB graphics card equipped with an I-5 5th generation processor connected to this microscope (Figure 2).

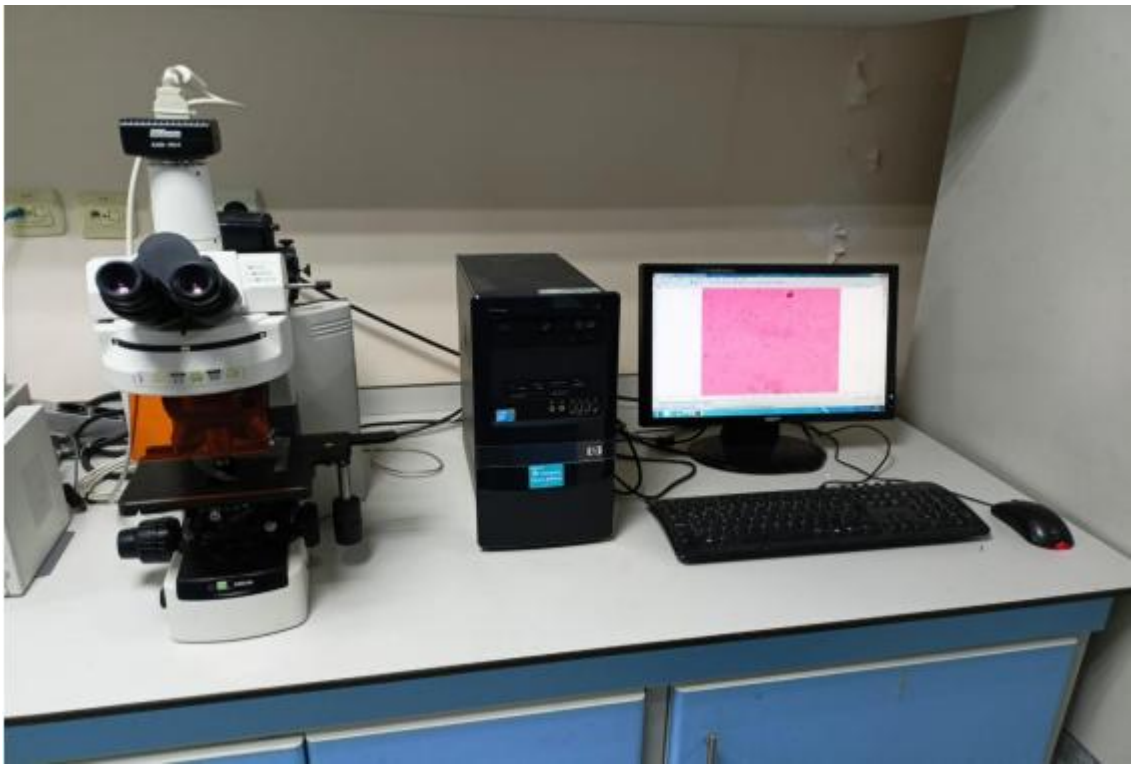


Figure 2. The light microscope and computer setup used for image acquisition for the dataset (Bağlar, 2023)

When capturing images with a light microscope, appropriate magnification and sufficient lighting adjustments are required at the optimal layers.

3. Methods

Digital image processing is a widely used method in image processing applications, encompassing processes such as image enhancement, noise reduction, restoration, encoding, and compression. Digital image processing can be applied in a wide variety of ways (Zhang et al., 2021). Due to the universality and flexibility of digital image processing, it does not involve complex measurement steps, which reduces learning costs. The field of microorganism analysis often requires expensive equipment to ensure the accuracy of measurement results. Digital image processing helps reduce these high costs (Ekstrom, 2012).

3.1 Developed Counting Methods

The Microorganism counting process consists of five stages. These stages include microbiological data collection, acquisition of microscopic images, image preprocessing, and evaluation (Zhang et al., 2021).
Microorganisms can be classified into seven categories based on various application areas such as agricultural, environmental, industrial, medical, water-related, and others. Appropriate samples of microorganisms present in the study area are collected. Subsequently, microscopic images are captured using suitable imaging devices connected to a computer (Gmür et al., 2000).
Preprocessing removes noise from images to enhance the contrast between object particles and the background. Preprocessing steps such as microscopic image scanning and electronic transmission can generate noise when scanning images of microorganisms. Image processing techniques can be used to reduce noise or improve image quality. Median filtering, average filtering, and Gaussian filtering are the primary techniques used to remove unwanted noise from images (Li et al., 2020). Microorganism counting is performed after these steps are completed.

3.2 Image Segmentation

Image segmentation is the most important method for identifying microorganisms. The extraction of a desired region involves the segmentation of the colony area (Zhang et al., 2021). According to Levner and Zhang (2007), image segmentation uses specific features visible on the foreground as references to separate an object from the background. In image processing-based microorganism counting methods, image segmentation is used to separate adjacent colonies and accurately determine their number. There are three main image segmentation methods: threshold segmentation, edge detection, and region-based segmentation (Perez and Gonzalez, 1987).

3.3 Circular Hough Transform (CHT)

Equation 1 represents a circle in the image, where (a, b) are the coordinates of the circle's center and r is the radius. In this case, a random edge point (x_i, y_i) will transform into a vertical circular cone in the parameter space (a, b, r). Murillo-Bracamontes et al. (2012) note that if all points in an image lie on a circle, the cones will intersect at a single point corresponding to the circle's parameters (a, b, r). This forms the basis of the circle comprising all points in the image.

$$(x-a)^2 + (y-b)^2 = r^2 \quad (1)$$

If the circles in an image have a known radius R, the search process can be reduced to 2D. The goal here is to find the (a, b) coordinates at the center of each circle. Each point on the circle is represented as a circle in Hough space with coordinates (x, y). The point where the circles with coordinates (a, b) and (x, y) intersect in Hough space is considered the center of all these circles. In this way, only three points of the circle can be represented, and this is done in conjunction with all pixels along the circle's edge. It is possible for a few pixels around the circle to be spurious. These spurious points are represented as circles with coordinates (a, b) in the central space and do not nearly coincide at a common center in Hough space. This method even allows for the detection of several circles with similar radius values (Murillo-Bracamontes et al., 2012).

$$x = a + R\,cos\theta \quad (2)$$
$$y = b + R\,sin\theta \quad (3)$$

In Equations 2 and 3, θ denotes the angle made with the horizontal axis. Using these two equations, a transformation can be performed between the (x, y) and (a, b) coordinates.
If the radius is unknown, the positions of points in parameter space will illustrate the formation of a conical surface in parameter space for an (x, y) point. In this case, every point (x, y) on the circumference of the circle will form a conical surface in parameter

space. The point (a, b, R) in the plane is represented by the accumulation cell at the point where the greatest number of conic surfaces intersect. A circle with a different radius (r) will appear at each level. In addition, it is possible to find circles with an unknown radius (r) using a three-dimensional accumulation matrix (Murillo-Bracamontes et al., 2012).
After identifying the foreground features in the image, the Circular Hough Transform (CHT) is used to convert them into aggregator votes in parameter space or Hough space. Equation 1 represents a circle with radius r centered at (a, b) in a two-dimensional space. (x, y) are considered as the foreground pixels of the image. In this case, using the transformation space parameters (a, b, r) , a cumulative matrix based on the votes in the transformation space is constructed in a manner similar to the traditional Hough transform in Equation 1, and the local maxima are accepted as the parameters of the circles in the image. If the image contains a large number of small particles across a wide range of particle sizes, significant issues will arise in real-time applications, such as the need for large amounts of memory, computationally expensive operations, and the inability to properly identify small particles (Mirzaei and Rafsanjani, 2017). Certain preprocessing techniques and modifications to CHT can improve the accuracy of the measurement results and increase the detection rate of smaller particles, which can reduce computational complexity. In this case, to reduce the computational complexity of 3D calculations, centers can be calculated in 2D, and in this scenario, radii will be calculated in 1D space (Yuen et al., 1990). In other words, circles with fixed radii are considered, and the true centers receive the highest score in the accumulator matrix. With this approach, the orientation of an edge point on the boundary of a circle is directed toward the center of the circle (Atherton and Kerbyson, 1999).

4. Results and Discussion

The computer used in the study was equipped with an Intel Core i7 10th-generation processor, 16 GB of RAM, and an 8 GB graphics card. The program, running Matlab R2021a, performed both the user interface and coding operations. Figure 3 shows screenshots of the program's user interface.

Figure 3. Screenshot of the microorganism counting program after counting (Bağlar, 2023)

In Method 1, 170 images obtained from 22 sample specimens using a light microscope were calculated separately with accuracies of 0.9 and 0.5. Additionally, counts were performed on the same images using Method 2, and the results are shown in Table 2. The developed application produced results by comparing image counts performed using two different methods with counts performed manually by an expert. The figures and tables illustrating the study's results were examined under separate headings.

Table 2. Table of data from the counting process obtained by a manual expert, Method 1, and Method 2 (Bağlar, 2023)

Number of Spores (Count)												
No.	**SAMPLE NAME**	**COUNTING METHOD**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	**Penicillium sp.**	**Manual Expert Count**	643	682								
		Method 1-0.9 Accuracy	1784	3024								
		Method 1-0.5 Accuracy	695	786								

		Method 2	119	190								
2	**Penicillium sp.**	**Manual Expert Count**	94	48	105	98	101	277	184	405	110	185
		Method 1-0.9 Accuracy	505	138	606	255	308	708	313	733	173	299
		Method 1-0.5 Accuracy	88	50	95	66	98	298	162	416	106	196
		Method 2	290	78	185	191	173	397	214	431	120	224
3	**Penicillium sp.**	**Manual Expert Count**	298	267	658	824	156	278	209	410	276	696
		Method 1-0.9 Accuracy	963	2011	1648	1409	537	465	557	917	916	1615
		Method 1-0.5 Accuracy	271	303	687	857	169	317	216	444	299	771
		Method 2	448	422	743	722	338	240	313	481	429	744
4	**Aspergillus niger**	**Manual Expert Count**	306	124	115	134	103	182	13	28	27	145
		Method 1-0.9 Accuracy	659	257	846	674	287	293	33	30	27	258
		Method 1-0.5 Accuracy	329	133	99	112	103	179	13	26	21	148
		Method 2	363	142	165	180	69	174	25	28	23	157
5	**Penicillium sp.**	**Manual Expert Count**	340	320	155	151	115	171	150	140	197	181
		Method 1-0.9 Accuracy	1110	439	179	178	199	186	181	174	338	309
		Method 1-0.5 Accuracy	352	319	155	157	117	167	154	141	199	189
		Method 2	325	292	148	147	153	151	115	137	191	147
6	**Cladosporium sp.**	**Manual Expert Count**	93	16	24	68	26	18	29	86	40	54
		Method 1-0.9 Accuracy	235	27	39	215	39	32	83	224	64	279
		Method 1-0.5 Accuracy	38	3	19	45	22	18	16	46	13	34
		Method 2	54	15	23	35	17	19	34	56	30	48
7	**Cladosporium sp.**	**Manual Expert Count**	91	45	54	39	51	41	53	141	36	148
		Method 1-0.9 Accuracy	311	156	83	50	52	81	71	209	115	274
		Method 1-0.5 Accuracy	97	44	33	22	42	39	28	82	29	134
		Method 2	55	43	49	31	40	50	31	107	15	69
8	**Penicillium sp.**	**Manual Expert Count**	84	93	44	48	110	120	141	105	36	79
		Method 1-0.9 Accuracy	175	172	69	166	124	219	159	188	58	87
		Method 1-0.5 Accuracy	84	118	29	28	91	109	91	101	31	41
		Method 2	79	107	34	43	78	117	104	68	31	59
9	**Aspergillus sp.**	**Manual Expert Count**	113	196	412	420	300	350	212	140	208	102
		Method 1-0.9 Accuracy	515	378	919	765	776	625	298	439	460	160
		Method 1-0.5 Accuracy	131	188	267	313	249	297	195	180	237	81
		Method 2	111	213	343	380	303	318	158	129	215	61
10	**Aspergillus sp.**	**Manual Expert Count**	51	28	60	26	120	96	55	75	36	14
		Method 1-0.9 Accuracy	343	43	126	126	161	124	73	227	54	13
		Method 1-0.5 Accuracy	55	13	49	24	86	80	34	76	29	5
		Method 2	12	10	16	19	30	28	19	28	13	4
11	**Penicillium sp.**	**Manual Expert Count**	18	26	41	22	27	32	25	24	14	26
		Method 1-0.9 Accuracy	24	35	44	30	27	32	25	24	14	28
		Method 1-0.5 Accuracy	18	29	30	20	24	20	21	20	13	22
		Method 2	14	24	29	20	26	26	25	24	15	18
12	**Penicillium sp.**	**Manual Expert Count**	124	95	68	22	41	44	25	23	21	243
		Method 1-0.9 Accuracy	162	154	124	49	76	60	30	33	31	371
		Method 1-0.5 Accuracy	94	65	68	21	40	41	15	17	21	266
		Method 2	79	58	47	31	41	34	15	9	14	133
13	**Penicillium sp.**	**Manual Expert Count**	54	76	71	83	28	30	108	46	34	25
		Method 1-0.9 Accuracy	163	299	279	217	50	50	220	91	59	46
		Method 1-0.5 Accuracy	48	68	58	44	16	17	78	38	16	21
		Method 2	31	72	58	65	17	19	62	28	22	21
14	**Penicillium sp.**	**Manual Expert Count**	75	93	112	138	696	718	269			
		Method 1-0.9 Accuracy	195	198	770	277	1005	1,212	650			
		Method 1-0.5 Accuracy	35	40	63	238	754	780	258			
		Method 2	47	46	118	112	295	417	187			
15	**Penicillium sp.**	**Manual Expert Count**	114	220	160	107	130	286	87			

		Method 1-0.9 Accuracy	409	560	310	277	328	969	217			
		Method 1-0.5 Accuracy	126	199	157	102	130	259	87			
		Method 2	178	250	193	171	175	375	114			
16	**Aspergillus sp.**	**Manual Expert Count**	351	155	252	350	121	96	86			
		Method 1-0.9 Accuracy	1488	309	384	669	247	163	163			
		Method 1-0.5 Accuracy	383	161	258	350	123	99	99			
		Method 2	278	144	234	293	105	76	76			

4.1. Results of Method 1

Figure 4 shows the flowchart used for microorganism counting in Method 1. The image loaded into the program first calculates the minimum and maximum radius values based on the sensitivity value. Then, the boundaries of the microorganisms are detected using the Circular Hough Transform. The end-user visually displays the boundaries of the microorganisms, identified using red circles in Figure 5(c). The program then counts the total number of microorganisms and displays the result as text at the top of the image.

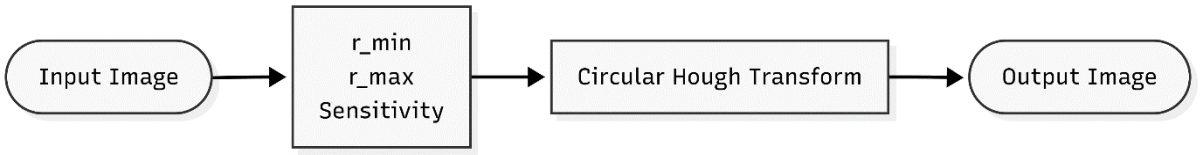


Figure 4. Flowchart for Method 1 from the application developed for microorganism counting (Bağlar, 2023)

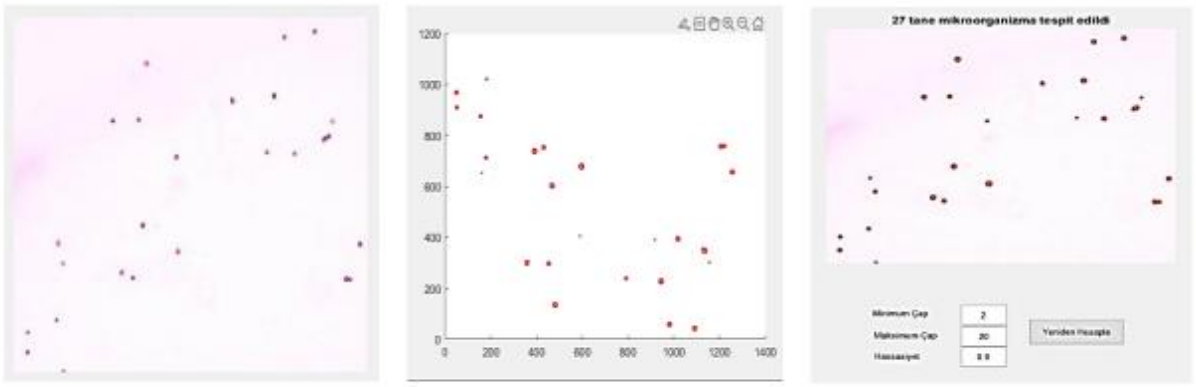


Figure 5. (a) Original image (b) Image with the Circular Hough Transform applied (c) Image showing the detected microorganisms with their edges outlined in red circles and the count is presented to the user (Bağlar, 2023)

4.2. Results of Method 2

The microorganism image selected in the main program is also selected for Method 2. The initial image acquired in RGB format is first converted to grayscale. A first-order image threshold was determined in the second stage of the grayscale conversion process. Subsequently, a binary image is created by setting the image threshold. Brightness values are used as an adaptive threshold and are known as a general image threshold. After correcting the fine structures along the image boundary, morphological opening is performed on the binary image using the necessary configuration elements. The erosion that occurs following an expansion using the same configuration element for both operations is known as the morphological opening process. Figure 6 shows the flowchart of the Method 2 application program.

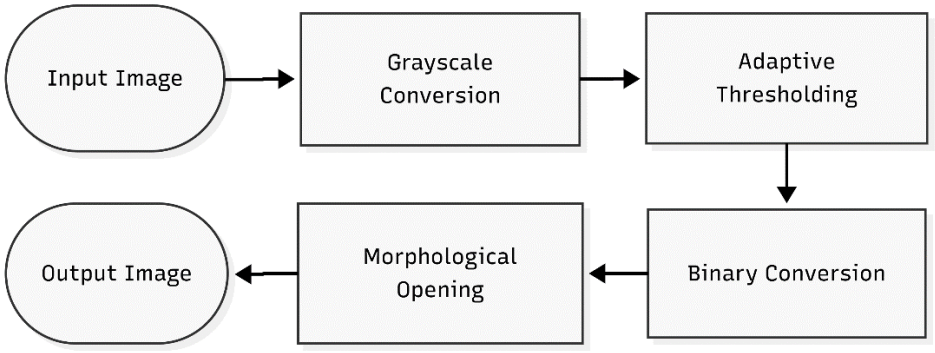


Figure 6. Flowchart for Method 2 from the application developed for microorganism counting (Bağlar, 2023)

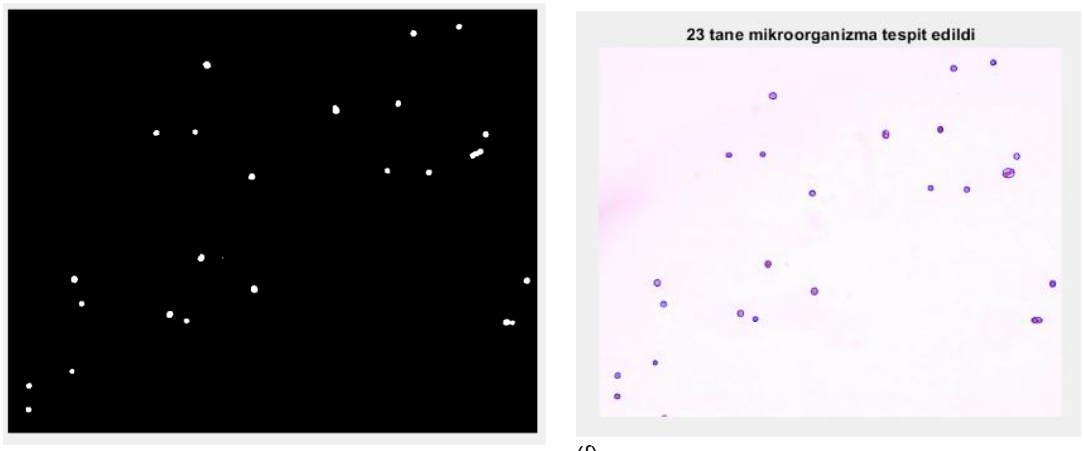
In Figure 7(f), the boundaries of the detected microorganisms are visually outlined with blue circles for the end-user. Additionally, it numerically displays the count of the detected microorganisms.

(a)

(b)

(c)

(d)



(e) (f)

Figure 7. (a) Original image (b) grayscale version of the original image (c) output of the detected image threshold (d) binary image obtained from the image using the threshold value (e) output with minor structures along the image boundary corrected (f) image presenting the detected number of microorganisms to the user by outlining their edges with blue circles (Bağlar, 2023)

5. Conclusions

A dataset was created using 170 images obtained from 22 microorganism specimens prepared using a light microscope for the study. Counting was performed on 170 of the obtained microorganism photographs. A specialist biologist manually performed four different counting procedures using Method 1 and Method 2 at sensitivities of 0.5 and 0.9. The results of the manual counting performed by a specialist biologist were compared with those of the new system. There is a correlation between the results of the manual counting and the applied techniques. The results closest to the manual counting were obtained with Method 1 at a precision of 0.5 and a correlation coefficient of 0.98.

When capturing images with a light microscope, some images may be affected by photography errors related to light and layer settings. Therefore, it may be difficult to identify and count microorganisms using both the manual count performed by an expert and the developed methods. When faced with such issues in the images to be analyzed, retaking the images under more suitable conditions using a light microscope—by adjusting parameters such as light and depth of field—will largely eliminate this problem.

Acknowledgements

This study is derived from the master's thesis of the first author, conducted under the supervision of the second author. The authors would like to thank the University-Industry-Public Cooperation Development Application and Research Center (ÜSKİM) at Kahramanmaraş Sütçü İmam University for providing the microfungi collection and laboratory facilities. We also gratefully acknowledge the Scientific Research Projects Coordination Unit of Kahramanmaraş Sütçü İmam University for supporting this study (Project Number: 2021/1-4 YLS).

Funding: This research was funded by the Scientific Research Projects Coordination Unit (BAP) of Kahramanmaraş Sütçü İmam University, under grant number 2021/1-4 YLS.

Conflicts of Interest: The authors declare no conflict of interest.

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