Bacterial Colonies on Agar Plate Counting Using Image Processing

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***Abstract*—Many pathogenic bacteria could cause human diseases. For example, the important diseases are pneumonia and foodborne illnesses, which could be caused by *Pseudomonas* sp. and *Salmonella* sp. respectively. Direct bacteria counting methods from blood or body fluid are the best methods that could give crucial information regarding the health situation of a person, a specialist oversees these methods. For the direct counting, an error could occur from eye fatigue. In this research, image processing was applied to automatic bacteria colony counting. This method can reduce the error. In this research, MATLAB software was applied to bacterial colony counting. *Salmonella enterica, Staphylococcus aureus, Escherichia coli, Serratia marcescens,* and *Pseudomonas aeruginosa* were selected as samples. This method had an accuracy of more than 90% and could reduce human error and improve counting efficiency.**

***Index Terms*—bacterial colonies, counting, image processing**

I. Introduction

Bacteria could be found everywhere. Normally, the size of bacteria is around 0.5-2 micrometers, which could not be seen by naked eyes. If bacteria are cultured on a solid media such as Nutrient agar and Luria-Bertani (LB) agar, bacteria will segment by binary fission until around 106 cells, called a bacterial colony. The bacterial colony on solid media could be seen by naked eyes. The size of a bacterial colony is around pinpoint to 5-8 millimeters. Each type of bacterial colony has a different size, consistency, texture, color, and pigment [1].

Counting bacterial colonies is usually used in microbiological analysis to estimate the number of bacteria. The counting is mostly used in the diagnosis of food, drug safety tests, environmental monitoring, public health, etc. [2]. For humans, direct counting bacterial methods requiring whole blood or body fluid are the best methods that give a piece of crucial information regarding the health situation of a person. Bacterial colonies counting on agar plates is a method to estimate the concentration of bacteria [3]. This method is a manual process with a specialist overseas. For direct counting, an error could occur because of eye fatigue. At present, a bacterial colony counter is used in many laboratories, but it is not completely automatic because a specialist has to use a probe to identify each colony [2]. These days, image processing plays an important role in this field of automatic counting.

There is a lot of research about counting bacterial colonies. For example, Xu, H. *et al.* used various types of ATP bioluminescence to compare with the colony counting method (manual counting) for checking surface cleanliness. ATP bioluminescence is used to detect bacterial colonies more clearly, but the result of this research, there are low correlation coefficients between 2 methods because the ATP bioluminescence method had several limitations for their study. The ATP bioluminescence method detects not only microorganisms, but also organic materials that are unsuitable for testing cleanliness [4]. Chiang *et al.* used a CCD camera and blacklight to capture bacterial colonies on the agar plate. They used image processing on the MATLAB program to analyze images. This method has an error of 3.3%. In addition, Zhu *et al.* used a near-infrared illuminator and near-infrared camera to capture images of bacterial colonies on agar plates. They also used MATLAB image processing to analyze the image. This method has an error of around ± 8 % and used time for counting around 10-20 seconds. That around 15-75 % of the time used in manual counting depends on the number of bacteria. [2], [5]

In this research, MATLAB software was applied to bacterial colonies counting. This method has used the algorithm in MATLAB to process images and detect bacterial colonies. The processed images have clearer colonies than the original image. As a result, the algorithm could detect the bacterial colony and increase the efficiency of colony counting.

II. Materials and Methods

A. Preparing Bacterial Colonies Sample

Salmonella enterica, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Serratia marcescens were selected as a sample. All bacteria were cultured in Luria-Bertani (LB) broth 5 milliliters and incubated in an incubator shaker at 37 °C for 16 hours. Then the ten-fold serial dilution was used for dilution the bacteria in LB broth. The concentration around 10^{-7} was used in this research [6], [7]. The spreading technique was used for spreading the bacteria on the LB agar plate and culturing all bacteria on the agar plate in an incubator

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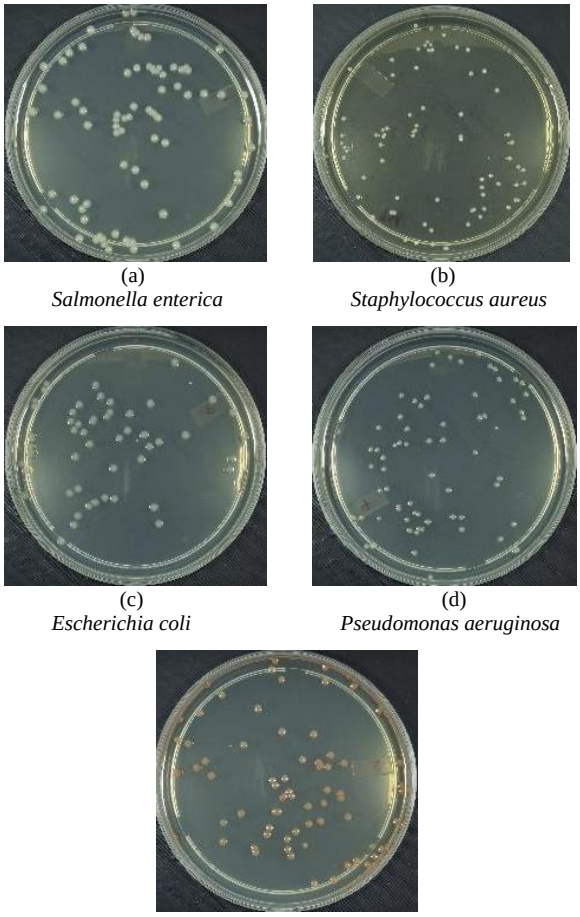
at 37 °C for 16 hours. Fig. 1 depicts the outcome of incubating the ager plates.



(a) *Salmonella enterica* (b) *Staphylococcus aureus* (c) *Escherichia coli* (d) *Pseudomonas aeruginosa* (e) *Serratia marcescens*

Figure 1. Show bacteria after incubating concentration 10^{-7}.

All the culture plate samples were captured by using a webcam camera, which has a high quality glass lens, a resolution of 1920×1080 pixels and auto focus function. The distance between the camera and plate must be fixed to the same size of the image's colonies. The dark background was used for contradistinction with colonies of bacteria on the culture plate.

B. Image Collection System

The image of bacterial colonies was taken by a webcam camera in the black box and 2 light sources. The black box is used for reducing the reflective light [2] and the size of the box is 20 × 20 × 30 centimeters. A Webcam camera with 2 megapixels was installed on the top of the box. The light sources were installed on the side of the box. The box's ground has a platform for placing the agar plate in the same position. The computer is used for capturing images and processing the images. The schematic diagram of the image collection system is shown in Fig. 2.

C. Image Processing and Detection

In this research, algorithms in the Matlab program were used for processing images of bacterial colony plates.

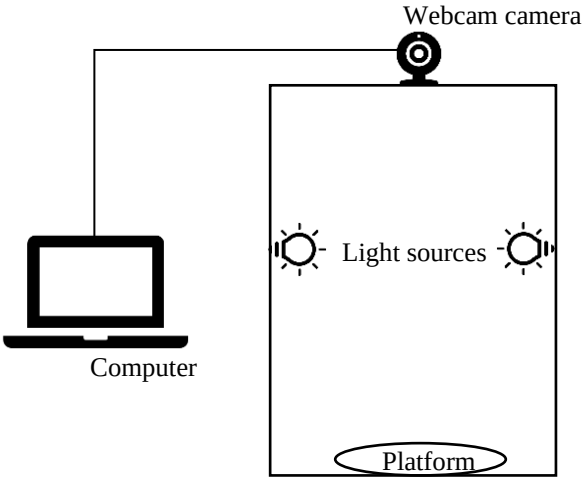


Figure 2. Schematic diagram of the image collection.

Create Mask algorithm used for creating a binary mask image from the Region of Interest (ROI). The pixels inside the ROI are white color (255 = white color) and outside are black color (0 = black color). This algorithm is used to create a binary image for subtraction with the original image. After subtraction, the inside ROI shows the original image and the outside shows black color.

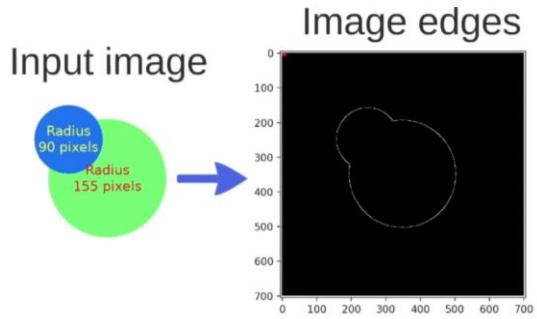
The Image Segmentation algorithm is the process of separating interesting objects from the image. This algorithm used the color or intensity in each pixel to separate the object and another. This process is more efficient and simplifies analyte or detecting object in image [8].

Imfindcircles is an algorithm for finding circles in images that is based on a Circular Hough Transform (CHT). CHT is good for finding the circles in the image that has noise, varying illumination, and occlusion.

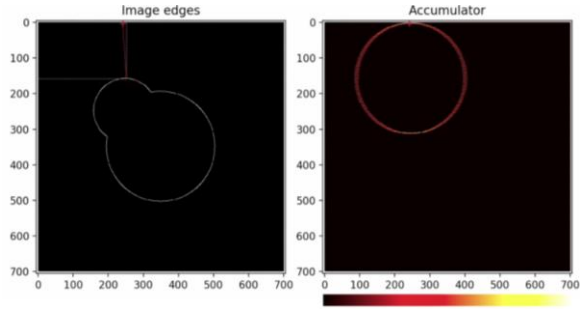
The CHT method has 3 steps for finding the circles. First, define the radius of a circle for detecting the circle. For example, the radius is 155 pixels. Second, the edges of the object are detected by the pixels that have difference when compared with surrounding pixels that are shown in Fig. 3(a). The coordinate pixel of the edge is collected in an accumulator array. Each edge coordinate pixel is assigned to a circle center. The coordinate pixels around the center, the distance be equal the radius that fixed in first step, the coordinate of circumference is collected in accumulator array. For the last step, when the accumulator array has all coordinated pixels around the pixel edge. The maximum value of the coordinate of circumference is the exact coordinate of the circle center that is shown in Fig. 3(c) [9].

Imfindcircles develops from CHT that consist of 2 main algorithms for finding circles. First is Phase-Coding and second is Two-Stage. Both algorithms have 3 common features. (1) Use of 2-D Accumulator Array, both algorithms solve the problem about large storage requirements and long processing times from the classical CHT. Both algorithms solve the problems by using a single 2-D accumulator array for all radii. (2) Use of Edge Pixels, this algorithm collects only pixels of high gradient in the accumulator array. So, the memory

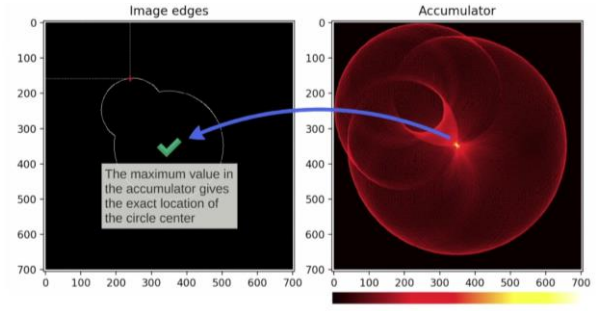
requirements are less than CHT and the speed is higher than CHT. (3) Use of Edge Orientation Information, this algorithm limits the number of bins that available to candidate pixels and limit time. This algorithm could optimize the performance for the method [10].



(a) Edge of image, from compare pixel and surrounding pixels.



(b) The circle's drawn from each pixel of image edge.



(c) The maximum value point from accumulator is the real center of circle.

Figure 3. Process of Circular Hough Transform (CHT) [9].

The process of image processing and detection has 6 steps that are shown in Fig. 4. The raw image from the camera has the uninteresting area that is shown in Fig. 5(a). The imfindcircles function was used in this study to find the largest circle that is the region of an agar plate with a radius of around 1000 – 1250 pixels.After getting the region of agar plate, the CreatMask function was used to create a binary mask image from the Region of Interest (ROI). The pixels inside the ROI are true (255) and outside are false (0) as shown in Fig. 5(b). Then subtraction between figure 5(a) and 5(b) the result that is shown in Fig. 5(c).

After cropping the image, the createMask function was used to adjust the color threshold of the image to remove the color of the agar plate that is shown in Fig. 5(d). The imfindcircles function was used again for find the colonies from the image. The imfindcircles function sets a radius of around 20 – 100 pixels to find the colonies. And the last collected the number of colonies and showed the result. The result is shown in Fig. 5(e).

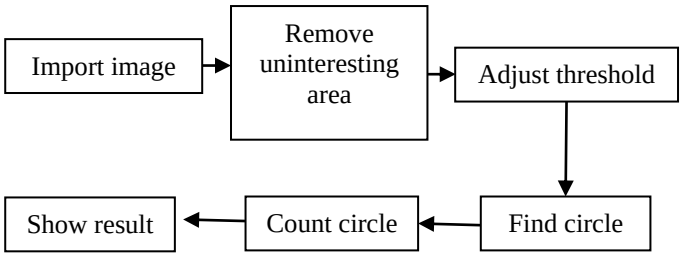
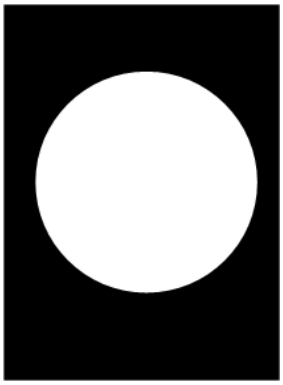


Figure 4. Process of image processing and detection.

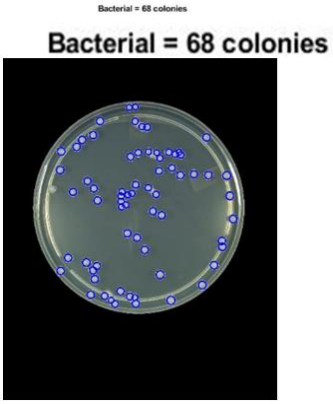
(a) Original image



(b) Find edge of plate and create ROI

(c) Subtraction between (a) and (b)

(d) Adjust color threshold of the image used CreateMask



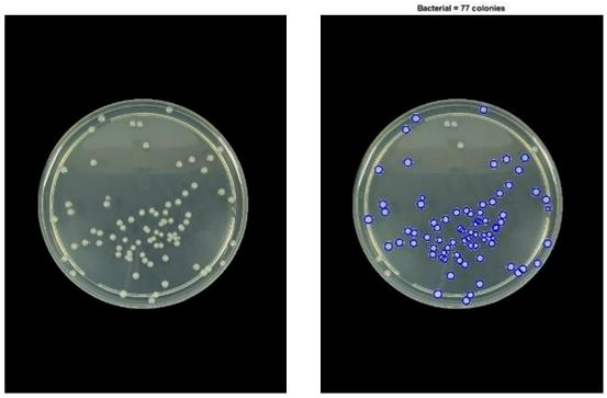
(e) find and count bacterial colonies

Figure 5. Image processing and detection.

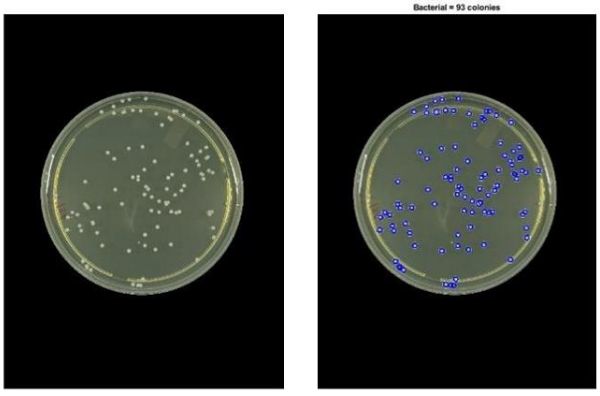
III. Result

The results of bacterial colony counting on agar plate by using image processing compared with counting by human eyes. For this research, the student of biomedical engineering, who studied and researched in microbiology for 3 years, counted the bacterial colony repeated 3 times per plate. Some results show in Fig. 6. The Fig. 6(a) shows the image of Salmonella enterica. There are 81 colonies for counting by human eyes and 77 colonies for counting by using image processing. The Fig. 6(b) shows the image of Staphylococcus aureus. There are 92 colonies for counting by human eyes and 93 colonies for counting by using image processing. The Fig. 6(c) shows the image of Escherichia coli. There are 33 colonies for counting by human eyes and 30 colonies for counting by using image processing. The Fig. 6(d) shows the image of Pseudomonas aeruginosa. There are 63 colonies for counting by human eyes and 58 colonies for counting by using image processing. The Fig. 6(e) shows the image of Serratia marcescens. There are 92 colonies for counting by human eyes and 94 colonies for counting by using image processing.

For all results of colony counting by image processing compare with manual counting by human eyes. There are 10 image of bacterial colonies plate per each bacterium. An error of *Salmonella enterica*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *and Serratia marcescens* is -9.95%, -2.24, -9.24, -9.14, and 1.62, respectively that show in Table I.

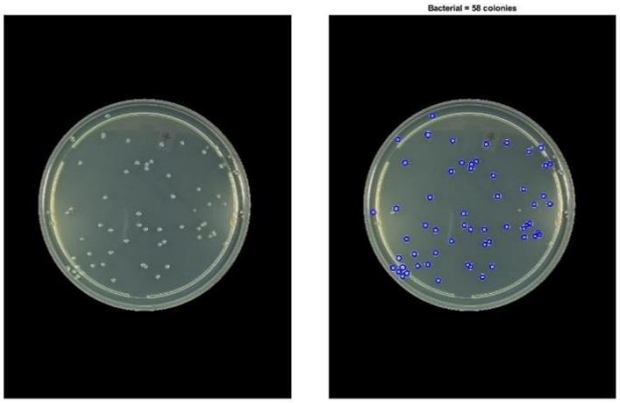


(a) Colony counting by human eyes 81 colonies and image processing 77 colonies of *Salmonella enterica*

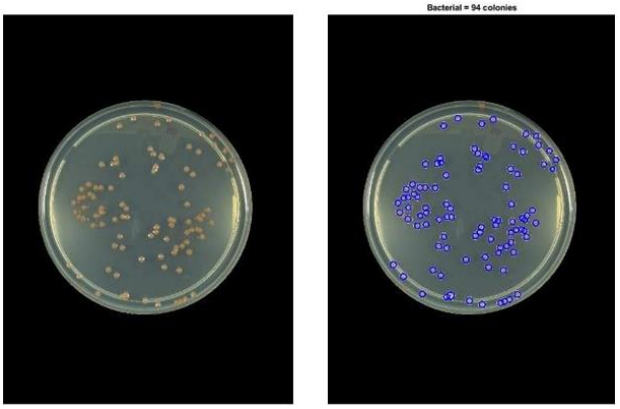


(b) Colony counting by human eyes 92 colonies and image processing 93 colonies of *Staphylococcus aureus*

(c) Colony counting by human eyes 33 colonies and image processing 30 colonies of *Escherichia coli*



(d) Colony counting by human eyes 63 colonies and image processing 58 colonies of *Pseudomonas aeruginosa*



(e) Colony counting by human eyes 92 colonies and image processing 94 colonies of *Serratia marcescens*

Figure 6. The result of counting between human eyes and image processing.

TABLE I. Results of Colony Counting by Manual (Human Eyes) and Image Processing

Bacterial	No. Plate	Counting bacterial (colonies)		Error (%)	Error average
		Manual (human eyes)	Image processing		
S. enterica	1	75	68	-9.33	-9.95
	2	73	65	-10.96	
	3	65	62	-4.62	
	4	72	67	-6.94	
	5	89	79	-11.24	
	6	92	82	-10.87	
	7	81	77	-4.94	

	8	74	70	-5.41	
	9	66	56	-15.15	
	10	85	68	-20.00	
S. aureus	1	69	63	-8.70	-2.24
	2	67	61	-8.96	
	3	107	106	-0.93	
	4	88	89	1.14	
	5	92	93	1.09	
	6	80	81	1.25	
	7	180	168	-6.67	
	8	76	78	2.63	
	9	83	82	-1.20	
	10	97	95	-2.06	
E. coli	1	33	30	-9.09	-9.24
	2	34	35	2.94	
	3	46	43	-6.52	
	4	30	27	-10.00	
	5	45	39	-13.33	
	6	47	44	-6.38	
	7	48	39	-18.75	
	8	55	45	-18.18	
	9	39	36	-7.69	
	10	37	35	-5.41	
P. aeruginosa	1	71	63	-11.27	-9.14
	2	52	47	-9.62	
	3	63	64	1.59	
	4	63	58	-7.94	
	5	62	61	-1.61	
	6	64	55	-14.06	
	7	63	49	-22.22	
	8	66	57	-13.64	
	9	73	66	-9.59	
	10	66	64	-3.03	
Serratia marcescens	1	76	76	0.00	1.62
	2	75	73	-2.67	
	3	72	71	-1.39	
	4	93	91	-2.15	
	5	93	95	2.15	
	6	73	72	-1.37	
	7	71	74	4.23	
	8	68	71	4.41	
	9	65	71	9.23	
	10	92	94	2.17	

IV. Discussion and Conclusion

When compared to manual bacterial colony counting with human eyes, automatic bacterial colony counting on agar plates using image processing from the MATLAB program had an average error of around 1.62 – 10%. The result of Serratia marcescens was the minimum error because Serratia marcescens colonies produce a red pigment. The red colonies stand out from the background and are easier to detect than the other bacterium. The automatic bacterial colony counting could count correctly more than 90 % and could reduce time for counting and estimating the number of bacteria. This method counts the colony in less than one minute, depending on the speed of the computer, which is used approximately 20-70% of the time from manual counting. This method had a result similar to the method of Zhu *el al.* [2] and still had lower accuracy for counting than the method of Chiang *et al.* [5] because Chiang's image of bacterial colonies on agar plate is clearer and could be detected correctly.

To develop the automatic bacterial colony counting on the agar plate, the light sources should be installed in a position that does not reflect on the agar plate. According to the findings of this study, when light sources reflect on agar plates, the colonies around that area fade in color and cannot be detected when the color threshold is adjusted. This method could be used to classify bacterial colonies in the future.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Sarinporn Visitsattapongse is responsible for research design, research summary, recommendation and contributes for data analysis. Manao Bunkum 's contrition is data analysis and research experiment.

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References

[1] P. Thomas, *Bacteria and Viruses*, 2004.
[2] G. Zhu, *et al.*, "Automated counting of bacterial colonies on agar plates based on images captured at near-infrared light," *J. Microbiol. Methods*, vol. 153, pp. 66-73, 2018.
[3] C. H. Collins, P. Lyne, J. Grange, and J. Falkinham, *Collins and Lyne's Microbiological Methods*, Lodon: Hodder Arnold, 2003, p. 480.
[4] H. Xu, *et al.*, "Evaluation of different detector types in measurement of ATP bioluminescence compared to colony counting method for measuring bacterial burden of hospital surfaces," *PLoS One*, vol. 14, no. 9, p. e0221665, 2019.
[5] P. J. Chiang, *et al.*, "Automated counting of bacterial colonies by image analysis," *J. Microbiol. Methods*, vol. 108, pp. 74-82, 2015.
[6] N. Vongmanee, A. Bunmak, and S. Visitsattapongse, "An automated colony counter for serial-dilution culture method," in *Proc. the 10th International Conference on Bioinformatics and Biomedical Technology*, Amsterdam, Netherlands, 2018, pp. 42-45.
[7] M. Bunkum and S. Visitsattapongse, "Discrimination colonies of *Staphylococcus aureus* and *Salmonella enterica* by using machine learning," *International Journal of Pharma Medicine and Biological Sciences*, vol. 10, pp. 30-34, 2021.
[8] S. Yuheng and Y. Hao. (2017). Image segmentation algorithms overview. [Online]. Available: https://arxiv.org/abs/1707.02051
[9] T. S. Körting. How circle hough transform works. [Online]. Available: https://morioh.com/p/fce0fd018299
[10] Imfindcircles. (March 2021). [Online]. Available: https://www.mathworks.com/help/images/ref/imfindcircles.html

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