Automatic Colony Segmentation on Agar Surface by Image Processing

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Received 30 May 2017; revised 24 December 2017; accepted 12 May 2018

The Medium (Petri dish, media, agar plate, petri culture, agar culture) are environments that have been formulated for the growth of microorganisms. These structures which are formed by reproduced microorganisms and can be seen by eye are called colony. Colonies formed on the agar, creating images of different morphological characteristics depending on the microorganism and growth media. Colony counting which is required in many applications in areas such as biotechnology and pathology is boring, time consuming and prone to human error process when the large number of colonies counted by hand. In this article, the sample images collected from dairies in the Marmara region are studied on and segmentation methods for separating images of microorganism colonies which is used in the dairy industry for the determination of microbiological analysis of products, have been investigated by the computer-aided image processing techniques such as Otsu, Multi-Otsu, Color K-Means, Watershed, Gabor Filters, Graph Based, Lossy Compression, Random Walker, Texture Filters with proposed comparison method. It was concluded that existing segmentation methods with appropriate parameters can be used to solve this problem and in comparison to other algorithms, Watershed segmentation algorithm has better performance values than others.

**Keywords:** Food Microbiology, CFU, Image Processing, Segmentation

### Introduction

Solid medium is a method that used for detection of microorganisms. They form different morphological features on the medium according to the environment and type of microorganism\(^1\). Image segmentation plays an important role in the solution of many problems. In the study of Quentin Geissmann\(^2\) circular colonies on agar surface is identified and counted with “direct thresholding” and “morphological segmentation” techniques. Euiwon Bae et al\(^3\) used region-growing segmentation algorithm to isolating circular colonies from images that formed by elastic light scatter. Silvio D. Brugger et al\(^4\) proposed a segmentation algorithm to discriminate circular bacterial colonies by their geometric shape properties. Er. Monita Goyal\(^5\) used image filtering, thresholding and Heywood circularity factor techniques to count of colonies that have specific sizes and circular characteristics. In this study, segmentation methods for separating images of microorganism colonies have been investigated and performance comparisons of the segmentation methods have been made to find efficient one by the computer-aided image processing techniques with proposed comparison method.

### Materials and methods

**Microbial detection by traditional methods**

For the detection of microbial indicators there are many traditional microbial detection methods such as solid medium, most probable number, membrane filtration, titer determination, rapid analysis based on metabolism, microscopic counts and biological stability test. There are advantages and disadvantages such as cost, time and applicability between these methods. In the food industry and many different areas such as biology, solid agar plate methods are widely used for the detection of indicator microorganisms\(^1\). The method comprising the following steps\(^6, 7, 8\):

- Pasteurized milk, yogurt, drinkable yogurt and water samples are analyzed to obtain images.
- Samples are diluted up to proper dilution decimally. 1 ml dilution is inoculated in a Petri dish.
- VRB Agar, Potato Dextrose Agar or Milk Plate Count Agar is prepared according to manufacturer’s instruction.
- For coliform detection, 10 ml VRB agar is poured, plate is swirled to mix and let solidify. Then 5 ml VRB agar is overlaid and let solidify again. Thanks to VRB agar containing neutral red
pH indicator, coli forms are formed pink colonies as lactose fermentation result.

- For mold and yeast, Potato Dextrose Agar is acidified with specific amount sterile tartaric acid before using. In this way pH is arranged to 3.5. At this pH, bacterial growth is inhibited.
- Milk Plate Count Agar is used for aerobic mesophilic bacteria to enumeration.
- Petri dishes are incubated 5 days at 25±2 °C for mold-yeast, 24 hours at 30±1 °C for coliform, 72 hours 30±1 °C for aerobic mesophilic bacteria.
- The image that formed at the end of the process is evaluated.

**Image acquisition**

Unfortunately, there is not any shared data source that can be used in application, so in order to creation of an image database, the work of collecting image of a Petri was performed in various milk processing factories that located in the Marmara region at Turkey. Samples were created under appropriate conditions using the ISO 6611/ IDF 94:2004 and IDF 73B:1998 standards. Photos were taken with 8mp camera, daylight and 3 point angular led lamp illumination and black background as Figure 1(a).

**Object segmentation**

Segmentation is the separation of the image to the region or category corresponding to the different object or parts of the object. Each pixel of the image is associated with a region. A good segmentation has the following characteristics:

- Pixels in the same region form a unified region that has similar values.
- Adjacent pixels in different regions have different values.
- The combination of all regions creates the image.
- The intersection of any two different regions is the empty set.

Segmentation is a critical step in image analysis, to work with objects in the image consisting of multiple pixels, each pixel is examined. The success of segmentation have the positive effects on the other stages of image processing but when the automatic segmentation algorithms are used, case specific successes are obtained and manual intervention is required to overcome problems in unforeseen circumstances. There are different approaches to segmentation such as thresholding-based methods, edge based methods and region-based methods.

**Thresholding based methods**

It is the simplest and widely used segmentation method. When a predetermined threshold value is t and a gray level value of the pixel at the position (i,j) is f_{ij}, if (1) condition is provided, the pixel is included in region 1, in other circumstances is included in region 2.

\[ f_{ij} \leq t \quad \text{... (1)} \]

In many cases, for separating the region of interest, the threshold value t is determined by the researcher by experimenting with different values. Pixels which are located in the same section (0 - t or t+1 - 255) are not always constitute a single connected object. Therefore, in the continuation of the process of thresholding, region merging or region splitting operations are applied to image. To separate the image into more than two regions, multiple threshold values can be used, the threshold value t can be calculated automatically by using information such as the gray level histogram graph of image. Otsu algorithm which is based on the thresholding-based methods is used to perform the automatic clustering-based image thresholding or the reduction gray level image to a binary image. In the algorithm, it is assumed that the image includes two groups of pixels associated with bimodal histogram (foreground pixels and background pixels) and an optimal threshold value which is make in-class variance minimum and inter-class variance maximum, is calculated to distinguish the two classes from each other. Unlike the original method, the version which uses multi-level threshold is referred to as multi-otsu.

**Edge based methods**

The edge filter is applied to the image and the detected discontinuity (sudden changes) in the image is called “edge”. According to the output of the filter process “edge” or “non-edge” labels are assigned to the pixels. Derivative operation is often used as an edge filter, first degree (2) and second degree (3)
derivative operations are mostly preferred. Canny, log, Prewitt, Roberts, Sobel are some of the most commonly used edge detection algorithms.

\[
\frac{\partial f}{\partial x} = f(x) - f(x-1) \quad \ldots (2)
\]

\[
\frac{\partial^2 f}{\partial x^2} = f(x+1) + f(x-1) - 2f(x) \quad \ldots (3)
\]

To form the outline of the object by using the detected edges, processes such as edge binding, model fitting (Hough transform), level sets, etc. is applied.\textsuperscript{9, 10, 11}

Region based methods

These methods try to group pixels that have similar value and adjacent, create linked objects from the pixels that in the same section, separate pixels that have different values. While the edge-based methods try to create objects by detection, merging and filling the object boundaries, region-based methods try to detect boundaries by starting from the center of object and with the outward growth. Regions can provide more information on the process of region determination because they can contain more pixels than edges. Algorithms such as merging, splitting, split-merge are used for the creation of the region.\textsuperscript{9, 10, 11}

Texture segmentation with Gabor filter which is a region-based method, is the process of zoning on the basis of image texture features. The discovery of the human visual system for multi-channel operation in the interpretation of textures has given the direction to the studies to use of Gabor filters for the detection of different texture regions on the basis of a multi-channel approach. Experience shows that Gabor filters is a reasonable model of simple cells in the mammalian visual system, therefore it is considered to be a good model of how the people are able to easily distinguish textures. Texture segmentation with Gabor filters consist of three basic stages:

- Designed Gabor filter array is applied to the source gray level image
- Feature extraction from the filtered image
- Clustering

Firstly, in order to extract Gabor magnitude, the image is converted to gray level by filtering with the designed Gabor filter array. For the use of the Gabor filter response in classification, Gabor features are obtained with the processes such as Gaussian smoothing, the addition of regional information to feature set, reshaping of the features by clustering algorithms and normalization. As a last step, the pixels are grouped into regions that representing the texture region with clustering.\textsuperscript{16, 17, 18, 19} In the K-means and color-based algorithm, images are converted to CIELAB color space from the RGB color space. In this way, when the brightness changes are negligible, the resulting 3 colors are provided to measure the visual differences. Segmentation is performed by the clustering with the K-means algorithm on color information in the “a *” and “b **” layer of the CIELAB color space\textsuperscript{20, 21, 22}. In the Watershed segmentation algorithm, when it is imagined that a surface drilled from local minima is immersed in water, starting from the lowest height minimum different basins will be filled by water step-by-step. If the dams are constructed to the meeting location of the water from two different basins, at the end of the immersion process each location is surrounded by completely dams to limit basin associated with this minimum. When the water level reaches the highest peak of the surface, this process is concluded. As a result, dams that separating the surface to different regions or basins are called basin lines or basins. Over-segmentation is the biggest drawback of the watershed transformation. Eliminating over-segmentation problem is to use a marker. Firstly, necessary features are defined to mark objects and they are called object pointers. Image parts that are known certainly not belong to any object is marked also and these are referred to as background markers. The remaining processes are the same for all applications. Gradient image is changed with the keeping the boundaries of region of interest that among the markers. Then, by using the watershed transformation on modified gradient image the determination of final frontier is performed.\textsuperscript{23, 24, 25} MATLAB environment has been used for coding and implementation of object segmentation algorithms and colony segmentation program. Colony Segmenter as Figure 3 (a) is designed to automatically segment petri images and compare segmentation algorithm’s performance. First of all, test images are manually segmented into colony objects and stored into “source objects” folder. Whole petri images are stored into “source images” folder also. Program needs this two folders path and a path for folder to store segmentation results. When the operation is started, algorithm makes segmentation on images by using all segmentation methods with all possible parameters step by step. To make a performance comparison of algorithms, we propose three parameters. To indicate
the target colony coverage ratio “TCCR“, for the predicted colony overflow ratio “PCOR“ and for incorrectly detected objects non-interest ratio “NIR“ parameters are defined. Calculations are made by comparing the target and the predicted object images. TCCR and PCOR vary between 0 and 1 and NIR vary 0 to \( \infty \). If predicted colony image totally cover the target colony, TCCR is become 1 and if the predicted image has not any extra part except the target, PCOR is become 1. So for perfect matching between target and predicted images the sum of TCCR and PCOR should become 2. In our performance comparison of segmentation algorithms, we use the average of TCCR+PCOR parameter. NIR is used as a secondary comparison parameter, ideal value for NIR is 0. Program store the best segmentation results with the algorithm parameters and TCCR, PCOR, and NIR results.

\[
TCCR = \left( \frac{\text{intersection area}}{\text{target area}} \right) \tag{4}
\]

Average \( TCCR = \frac{\sum_{i=1}^{n} TCCR}{n} \) \tag{5}

\[
PCOR = \left( \frac{\text{intersection area}}{\text{predicted area}} \right) \tag{6}
\]

Average \( PCOR = \frac{\sum_{i=1}^{n} PCOR}{n} \) \tag{7}

\[
NIR = \left( \frac{\text{non-interest number}}{\text{target number}} \right) \tag{8}
\]

Average \( NIR = \frac{\sum_{i=1}^{n} NIR}{n} \) \tag{9}

**Results and Discussion**

By experimenting with different lighting (top-bottom led illumination, fluorescent illumination, daylight) and flooring (black, white, illuminated) alternatives, it has been observed that less noisy photos (Figure 1 b, Figure 1 c) is obtained with daylight and 3 point angular led lamp illumination and black background. Additionally, photography is performed by Nikon D80 camera with 850 nm wavelength light (near infrared) but the results shows that this kind of lighting do not have any differences on molds, yeasts and bacteria colonies so a successful result could not be obtained for this illumination. Colonies of yeast and bacteria is able to create similar images with the medium. This similarity leads to lower the performance of thresholding based segmentation. As a result of the work that we do with the Otsu algorithm, the low threshold level has been found that it increase the noise level and damage the object images by merging (medium-object images) in the images of objects that are similar to the medium image. The use of large threshold level causes unable to determination of the colony images as shown in Figure 2 (a). Our studies show that, performance of image segmentation by automatic threshold detection without human intervention is low in the image where different colors and tones is located. Results that we have done using Gabor filters with parameters such as filter orientation, Gaussian smoothing coefficient, cluster number and distance metrics have good performance values although it has been observed that in the images with different textures; human intervention is required for the determination of the cluster number. When you run a clustering algorithm for fewer cluster number than the number of clusters in the image; colony merging and for more cluster number; colony splitting have been occurred. In addition, mold image that containing different texture regions is detected as more than one mold due to separating into multiple regions with Gabor segmentation as shown in Figure 2 (b). Object degradation directly affects the estimation of the type and count of objects so image segmentation with Gabor filters has a negative impact on system performance for images that has multiple textures or microorganism types. Separation of the objects from petri image should be done without human intervention and without suffering any degradation. Results of texture segmentation techniques with color based K-means algorithm with Squared Euclidean, City Block, Cosine distance measures and different cluster numbers such as 3, 4, 5, 6 (at least 3 clusters should be appeared on surface; background, agar and colony) shows that mold colony images that have different color zones are degraded after color based segmentation as shown in Figure 2 (c). In addition, when we run a K-means clustering algorithm for fewer cluster number than the number of clusters in the image; colony clustering algorithm for fewer cluster number than the number of clusters in the image; colony merging and for more cluster number; colony splitting have been occurred.

**Fig. 2 — Results, (a) Otsu algorithm yeast-media thresholding problem (b) Gabor segmentation result for multi-textured mold object (c) Color based segmentation result for multi-textured mold object**
The color number of colony images is not constant so the number of cluster cannot be estimated and human intervention is required. Examination of the resulting image shows that, for the separation of objects, most successful results was obtained by the “Watershed Segmentation” method (Figure 3 b, Figure 3 c) with algorithm parameters such as morphological opening by 18 size disk, morphological closing by 4x4 rectangle, image eroding by 4x4 rectangle. It is observed that the algorithm identified numerous regions that will be considered as non-counting objects except for mold, yeast and bacteria. These non-colony objects does not prevent the detection of molds, yeasts and bacteria colonies but increases the processing load by unnecessary objects that should be treated and arises the necessity of the identification of non-colony objects. The best segmentation result with Watershed algorithm (Table 1) has the 1.72 TCCR+PCOR value that is the maximum in all results and 4.6 NIR value that is the maximum in the best ranked TCCR+PCOR algorithms. Segmentation with Gabor filters which is the second good performance algorithm has the 1.29 TCCR+PCOR value and 2.20 NIR value. According to the experimental results, it is revealed that the Watershed segmentation algorithm significantly outperforms the other methods in terms of TCCR+PCOR and suitable to solve colony segmentation problem. Experiments show that parametric results are consistent with the visual comparison.

**Conclusions**

In comparison to the algorithm, the best matching with target object without human intervention is evaluated as performance criteria. In this study, different segmentation methods, different values of the parameters for this methods and automatic parameter selection techniques is used on images that containing molds, yeasts and bacteria colonies. Yeast and bacterial colonies can produce images that similar with media, this similarity causes to lower the performance of thresholding-based segmentation. For images with different textures some difficulties such as determining the number of clusters with human intervention, colony image merging with small number of clusters and colony image splitting with large number of clusters is determined. It is observed that mold image that containing different texture regions is detected as more than one mold due to separating into multiple regions and mold colony images that have different color zones are degraded after segmentation process. In our work for separating the images of mold, yeast and bacteria colonies, watershed segmentation method has higher success rates in terms of average TCCR+PCOR in comparison to other algorithms. Experiments show that, algorithm detects too much objects that are not a colony, for this reason, the results that produced should be subject to final assessment by microbiology specialist or intelligent computer systems. Colony counting which is required for many applications in industry, is boring, time consuming and prone to human error process when large number of colonies counting by hand. In addition to being long and laborious process the detection and counting of the colonies is
dependent on several factors such as staff’s fatigue, experience and ability that can vary in relation to the training and experience of laborant. To be able to counting colony, training and experience on colony morphology of laborant is required. This education and experiences cannot be transferred from person to person so this training and experiencing stages are required for all stuffs too. These requirements for identification and counting of the colonies reveal the need of automatically performing of these processes by a computer system. With the proposed system in this study, it is showed that microorganism colony images in agar surface image can be separated automatically without human intervention and this will contribute to the continuation of work such as industrial automatic petri photo capturing, storing and sharing systems and intelligent colony morphology decision making systems. In our feature works, to improve the performance of the segmentation system, we plan to develop intelligent segmentation methods for images that can contain mold, yeast and bacteria colonies. In addition, we plan to develop feature extraction methods to use in decision making system for automatic classifying of segmented images.

Acknowledgments
This research was financially supported by the Yalova University and Bursa Technical University.

Author contributions
Altuntas V. is the corresponding author, designed the research plan, developed the algorithms, participated in experiments, data analysis and the writing of the manuscript. Altuntaş S. is performed the experiments, participated in the data analysis and the writing of the manuscript. Gök M. is supervised the project, participated in the research plan.

References