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An Improved Automated Method for Identification of Bacterial Cell Morphological Characteristics

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Abstract *:* The main objective of the present study is to develop an improved automatic tool to characterize the morphology of bacterial cells in digital microscopic cell images. Geometric shape features are used to identify the different characteristics of bacterial cells, namely, flagellated and fimbriated. The current methods rely on the subjective reading of profiles by a human expert based on the various manual staining methods. In this paper, an improved automated method is proposed for bacterial cell characterization based on their different characteristics by segmenting digital bacterial cell images and extracting geometric shape features for cell morphology. An optimal feature set is identified, out of seven feature sets proposed. The classification techniques, namely, 3σ and K-NN classifiers are used to identify the bacterial cells based on their morphological characteristics. The experimental results are compared with the manual results obtained by the microbiology expert and demonstrate the efficacy of the proposed method.

Keywords : Bacterial image analysis, flagellum, fimbriae, bacterial cell morphology, automated image analysis, edge detection, 3σ classifier, K-NN classifier.

INTRODUCTION

Microscopical examination is usually the first step in the identification of an organism. The morphological features of importance include: size, shape, arrangement, presence of flagella and spore. The different types of microscopes are commonly employed for bacterial study, namely, optical or light microscope, phase contrast microscope, dark field illumination microscope, fluorescence microscope and electron microscope. Low or medium-power light microscopy is usually adequate for the study of fungi and protozoa, whereas oil-immersion microscopy is necessary for identification of bacteria.

Most of the bacteria are unicellular microscopic organisms which can only be seen through microscope. Bacteria exist in different sizes and shapes and they measure in micro-meter (which is a millionth part of a meter). Bacteria are found everywhere and in all types of environments. There are numerous types of bacteria in the world. Bacteria are mainly classified based on their shapes, biochemistry and staining methods. Recently, along with the morphology, other profiles such as their metabolic activities, conditions required for their growth, biochemical reactions, antigenic properties, and other characteristics are also helpful in classifying the bacteria. However, each type of bacteria has its own characteristics. Most of the bacteria are characterized by three main shapes: rod (rod shaped bacteria are called bacilli), sphere (sphere shaped bacteria are called cocci) and spiral (spiral shaped bacteria are called spirilla or spiral). Some bacteria posses different shapes, which are more complex than the above mentioned shapes.

The anatomy of bacterial cell structures possess, cell wall, cell membrane and the protective gelatinous covering outside the cell wall known as capsule. Apart from this, some bacteria possess filamentous appendages, flagella and fimbriae, which protrude from the cell surface. Surface structures originate outside the cell membrane, sometimes being attached to it, and extend into the environment [14]. The structure of bacterial cell is shown in Fig.1.

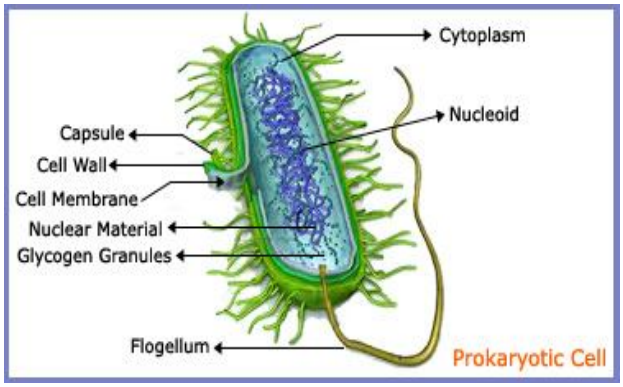

Fig 1: Structure of bacterial cell

In this paper, the images of bacterial cells of flagella and fimbriae surface structures are considered for the identification and classification of the cells. In our study, the two morphological structures, namely, flagella and fimbriae of bacterial cell structures are considered and are explained below:

Flagellum: Flagella are filamentous, cytoplasmic appendages protruding through cell wall. These are unbranched, long, thread-like structures composed entirely of protein (*flagellum*), 12-30 nm in diameter and 5-16 µm in length. They are the organs of locomotion and have characteristics patterns of distribution in the bacterial cell. There are four types of flagellar distribution on bacteria, (i) Monotrichous – single polar flagellum, (ii) Amphitrichous – single flagellum attached to each end, (iii) Lophotrichous – tufts or flagella at one or both ends and (iv) Peritrichous – numerous flagella all over the bacterial body. The sample flagellated bacterial cell structure is shown in the Fig. 2. Although chemical composition of flagella of different genera of bacteria is similar, they are antigenically different. Specific antibodies are produced in high titers in response to antigenic stimulation by flagella. Flagellar antibodies are useful in serological diagnosis but do not have any protective role.

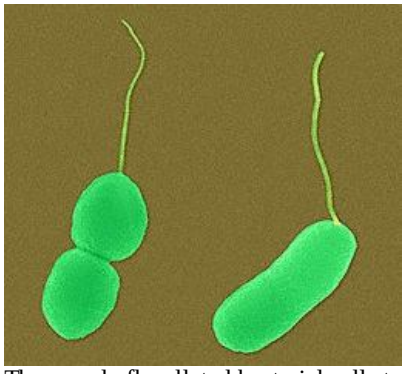

Fig 2: The sample flagellated bacterial cell structure.

Fimbriae: These are thin short filamentous appendages (0.1 to 1.5 µm long and less than 4 to 8 nm thick) extruding from the cytoplasmic membrane. They are also called pili. Fimbriae are found only in some Gram-negative bacteria and project from the cell surface as traight filaments. Each bacteria possesses 100-500 pili peritricoushly. They are more numerous than flagella. The fimbriae are best developed in freshly isolated strains from liquid culture. Gthey tend to disappear when subcultures are made on solid media. Fimbriae is composed of protein subunits called pilin. They are antigenic. Pili can be only seen under the electron microscope. The Fig. 3 shows the sample fimbriated bacterial cell structure . The most commonly occurrence of fimbria is in *enterobacteriaceae*, e.g., *Salmonella*, *Proteus* and *Shigella*, etc. There are 3 main types of fimbriae, common pili, sex or F (fertility) pili and col l (colicin) pili. Common pili are of 6 types based on their morphology, number per cell, adhesive properties and antegnic nature. The important function of pili or fimbriae are; (i) Adhesion – Pili are organs of adhesion on cells, (ii) Sex pili- These are specialised fimbriae and fewer in number, possessed by male bacteria and (iii) Haemaggulutination – Certain fimbriated bacteria strongly agglutinate red blood cells of guineapigs, fowl, horses and pigs. These are stained negatively by phosphotungestic acid (PTA).

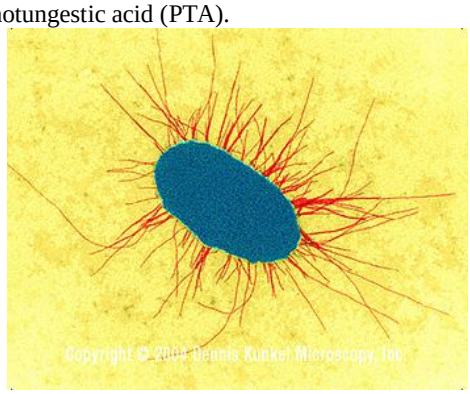

Fig 3: Sample fimbriated bacterial cell structure

The complete atomic model of the bacterial flagellar filament by electron cryomicroscopy has been carried out by Koji et al. [1]. The real-time imaging of fluorescent flagellar filaments has been done by Linda et al. [2]. The bacterial growth and motility in sub-micron constrictions has been investigated by Jaan et al. [3]. Hiremath and Parashuram [4] have investigated the automatic Gram-staining characterization of digital microscopic bacterial cell images using color and cell wall properties. A simple image analysis algorithm for evaluation of extended filaments length based on the enhanced digitized image using statistical analysis is proposed [7]. GSI of bacterial and archaeal cells in the natural microbial communities of slightly and extremely saline environments has been proposed [8]. Digital image analysis of actinomycetes colonies as a potential aid for rapid taxonomic identification has been investigated [9]. Characterisation of PHB storage in activated sludge extended filamentous bacteria by automated colour image analysis has been examined [10].

In this paper, the objective is to propose an improved method for automatic identification and classification of bacterial cell characteristics in digital microscopic images using geometric shape features that characterize the different morphology of bacterial cells. For identification and classification, 3σ and K-NN classifiers are used. The experimental results are compared with the manual results obtained by microbiology expert and demonstrate the efficacy of the proposed method.

MATERIALS AND METHODS

The strains were inoculated in broth media and incubated overnight at 37°C for 18 hrs in agitation rotor. The bacterial cells from each culture were recovered by centrifugation at 6,000 rev/min and the cells were washed twice with potassium phosphate buffer (50 mM, pH 7.0). Bacterial cells were then fixed by immersion in 2.5% glutaraldehyde in potassium phosphate buffer (50 mM, pH 7) overnight at 40C. Then the specimens were washed twice with buffer and dehydrated by an ethanol series (v/v) ranging from 30, 40, 50, 60, 70, 80, 90 to 100% and stored in 100% ethanol. For SEM, the specimens were dried to critical point, coated with gold and examined with an S-200C scanning electron microscope. The cell volumes and surface area were directly measured from Scanning Electronic Microscopy (SEM) photograph. The digital images of cells on the slides are captured by a digital camera interfaced with a PC and then stored in the disk memory for further processing and image analysis. For the purpose of experimentation, a datasets containing the 300 color images of bacterial cells(non-overlapping) with different characteristics, namely, flagellated and fimbriated, is prepared.

PROPOSED METHOD

The objective of the present study is to propose an improved method of automated image analysis of digital bacterial cell image in order to identify the different morphology of a bacterial cell: flagellated or fimbriated using lesser number of geometric features of cells and a better classifier such as K-NN classifier. Out of many geometric features used by various authors in the literature [5], [6], [13], it is observed that mainly seven geometric shape features, namely, elongated, circularity, eccentricity, tortuosity, length-width ratio, relative convex area and relative convex perimeter, are used and these are defined as given below:

Elongated(x_1): It is the ratio of longer side to shorter side of the bounding rectangle (Elongated = Length / Width).

Circularity(x_2): It is to measure irregularity of circular objects. Circularity = 4π(Area) / perimeter2

Eccentricity(x_3): It is the ratio between the lengths of the short axis to the long axis(Eccentricity = axis length $_{short}$ / axis length $_{long}$). The value of eccentricity is between 0 and 1. Eccentricity is

also called ellipticity with respect to minor axis and major axis of the ellipse.

Tortuosity(x_4): It is the ratio of contour length (perimeter) to the maximum linear length (MajorAxis Length) between any two points on the contour (Tortuosity = Major axis / perimeter)

Length-width ratio (LW ratio) (x_5): It is the ratio of the length of longest chord (Length) of the shape to the longest chord perpendicular to it (Width). (Length-width ratio = Major axis / minor axis).

Relative convex area(x_6): It is the ratio of convex area to area (Relative convex area = convex area / area).

Relative convex perimeter(x_7): It is the ratio of convex perimeter to perimeter (Relative convex perimeter = convex perimeter / perimeter).

Out of these features, it is essential to determine that combination of features which yields better classification results. The proposed method comprises the following steps, which are depicted in the block diagram shown in the Fig. 4:

- Preprocessing using morphological operations
- Segmentation using Canny's edge method
- Geometric feature extraction.
- Classification into flagellated or fimriated cells

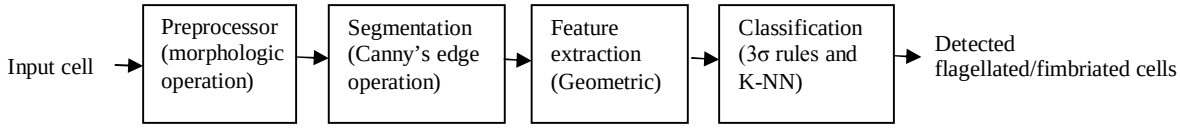

Fig 4: The block diagram of the proposed method

The bacterial cell images generally contain noise, small debris and artifacts depending on the different staining methods. To remove this debris, we have preprocessed the image by applying morphological operations. This stage is of high importance in achieving good results in segmentation and further process. The gray scale image of cells is segmented using the Canny's edge method, which yields binary image. After labeling the segmented image, the geometric features $x_i,\ i = 1,2,\dots,n$ are extracted for each labeled segment.

These features are used as a basis for the cell characteristics. Using the training set of images (with known cell classification), for each feature $x_i^k,\ i = 1,2,\dots,n$ of kth cell type, we compute the mean $\bar{x}_i^k$ and standard deviation σ_i^k of the sampling distribution of the feature values and store them as knowledge base. In the testing phase, for a given test image, feature values $x_i^{(test)}$ of the segmented regions (cells) are computed and then cell classification is done using the 3σ rule, namely: For a segmented region in the test image, if the feature values $x_i^{(test)}$ lie in the interval $\bar{x}_i^k \pm 3\sigma_i^k,\ i = 1,2,\dots,n$, then the region is a cell of type k. The k=1, 2 correspond to flagellated and fimbriated cell types, respectively.

The proposed method for the automatic identification of bacterial cell morphological characteristics, namely fimbriated and flagellated, using geometric shape features that characterize the different morphology of bacterial cells, are given below in the Algorithms 1 and 2.

Training phase:

Algorithm 1: Extraction of features for knowledge base

Step 1: Input bacterial cell image (RGB color training image).
Step 2: Convert the RGB image into gray scale image.
Step 3: Perform preprocessing using morphological operations
Step 4: Segment the resulting image of Step 3 using Canny's edge method.
Step 5: Perform labeling the segmented image.
Step 6: For each labeled segment, compute geometric shape features $x_i,\ i = 1,2,\dots,n$, for each cell type k. The k=1, 2 correspond to flagellated and fimbriated, respectively.
Step 7: Repeat steps 1 to 6 for all the training images.
Step 8: Compute mean $\bar{x}_i^k$ and standard deviation σ_i^k of the sampling distribution of the feature values for each cell type k and store them as knowledge base.

Classification phase:

Algorithm 2: Classification of bacterial cell morphology

Step 1: Input bacterial cell image (RGB color training image).
Step 2: Convert the RGB image into gray scale image.
Step 3: Perform preprocessing using morphological operations
Step 4: Segment the resulting image of Step 3 using Canny's edge method.
Step 5: Perform labeling the segmented image.
Step 6: For each labeled segment, compute geometric shape features$x_i,\ i = 1,2,\dots,n$, for each cell type k. The k=1, 2 correspond to flagellated and fimbriated, respectively.
Step 7: Apply 3σ rule and K-NN classifiers for classification of the bacterial cells: A segmented region is of cell type k, if its features $x_i^{(test)}$ lie in the interval $\bar{x}_i^k \pm 3\sigma_i^k,\ i = 1,2,\dots,n$ .The k=1, 2 correspond to flagellated and fimbriated, respectively.
Step 8: Repeat the Steps 6 and 7 for all labeled segments and output the classification of identified cells.

The Algorithm 2 can also be implemented using K-NN classifier instead of 3σ classification rule, where K=1 is minimum distance classifier. Further, it can be implemented using different combinations of features.

K-NN classifier

The K-nearest neighbor (K-NN) classification is performed by using a reference data set (training set) which contains both the input (feature set) and the target variables (known cells) and then by comparing the unknown (test data) which contains only the input variables (features) to that reference set. The distance of the unknown to the K nearest

neighbors determines its class assignment by either averaging the class numbers of the K nearest reference points or by obtaining a majority vote from them [15].

EXPERIMENTAL RESULTS AND DISCUSSIONS

For the purpose of experimentation, 300 color digital bacterial cell images containing different characteristics of bacterial cells (non-overlapping) namely, flagellated and fimbriated spiral are considered (as described in the section 2). The implementation is done on a Pentium Core 2 Duo @ 2.83 GHz machine using MATLAB 7.9. In the training phase, each input color image of bacterial cell (Fig. 5(a)) is converted into gray scale image, and the morphological operations such as erosion, reconstruction and dilation are applied [11], [12]. The resulting image is segmented using canny's edge method to obtain segmented binary image (Fig.5(b)). The segmented image is labeled and for each segmented region (known cells), the geometric features are computed. The Table 1 present the geometric feature values computed for the segmented cell regions of the image in Fig. 5(b) and 1(d).

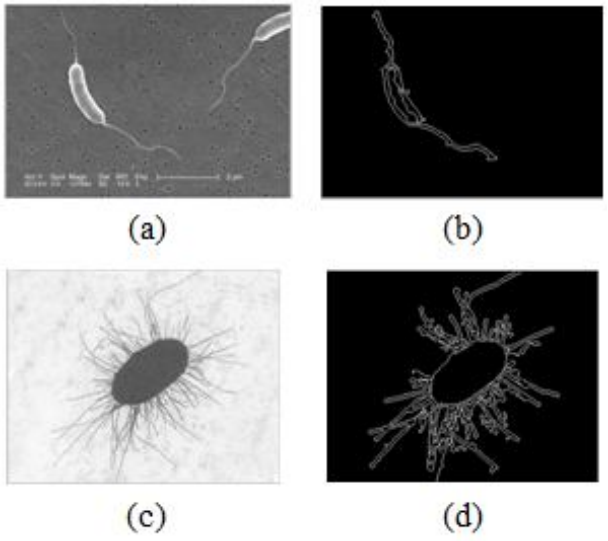

Fig 5: (a) Microscopic flagellated cell image, (b) Segmented image of image in (a), (c) Microscopic fimbriated cell image, (d) Segmented image of image in (c).

Table 1: The geometric feature values of the cell regions of the image in the Fig. 5(b) and (d)

Geometric cell features	Values of geometric features of bacterial cells	
	Flagellated (Fig.3(a))	Fimbriated (Fig.3(d))
Elongated (x_1)	1.2025	1.4016
Circularity (x_2)	0.0160	0.0024
Eccentricity (x_3)	0.9609	0.7090
Tortousity (x_4)	0.3304	0.0555
LW ratio (x_5)	3.6129	1.4180
Relative convex area (x_6)	0.2744	0.0136
Relative convex perimeter (x_7)	0.0663	0.0057

The mean and standard deviation of the sampling distribution of these features obtained from the training images are stored in the knowledge base of the cells: flagelatted and fimbriated, as shown in Table 2.

Table 2: Mean and standard deviation (SD) of geometric feature values of flagelatted and fimbriated bacterial cells

Geometric features	Flagellated cell		Fimbriated cell	
	Mean	SD	Mean	SD
Elongated	1.56137	0.4566	1.2060	0.1534
Circularity	0.01018	0.0037	0.0029	0.0004
Eccentricity	0.85817	0.1435	0.6551	0.1027
Tortousity	0.26886	0.0697	0.0605	0.0081
LW ratio	2.52099	0.8801	1.3609	0.1559
Relative convex area	0.13408	0.0961	0.0187	0.0036
Relative convex perimeter	0.03551	0.0171	0.0074	0.0011

In order to explore efficient feature set, the experimentation of the proposed algorithm is conducted by considering the different feature sets, namely, F1, F2, F3, F4, F5, F6 and F7 as defined below:

F1={x_1, x_3, x_4} = {elongated, eccentricity, tortousity}

F2={x_1, x_2, x_3} = {elongated, circularity, eccentricity}

F3={x_3, x_4, x_5} = {eccentricity, tortousity, LW ratio}

F4={x_4, x_5, x_6} = {tortousity, LW ratio, relative convex area}

F5={x_1, x_3} = {elongated, eccentricity}

F6={x_1, x_2, x_3, x_4, x_5} = {elongated, circularity, eccentricity, tortousity, LW ratio}

F7={x_1, x_2, x_3, x_4, $x_{5, x6, x7}$} = {elongated, circularity, eccentricity, tortousity, LW ratio, Relative convex area, Relative convex perimeter}.

It is found that the feature sets F1 and F4 yield better classification results compared to the other feature sets. The classification results obtained for each of the feature set, using different values of K in the K-NN classifier, are presented in the Table 3.

In the testing phase, for a test image, the feature extraction algorithm is applied and the test feature values $x_i^{(test)}$ for each segmented region are used for classification using 3σ rule and K-NN classifier. The classification results are given in the Table 3. For testing images, the 3σ classifier has yielded an accuracy of 84.75% to 92.5%. The sample misclassification results are shown in the Fig.6. In the Fig. 6(d), it is observed that poor quality of segmentation has led to the misclassification, whereas in Fig. 6(b), the 3σ classifier is not good enough to classify it correctly. Hence, there is a need to improve both segmentation and classification methods to obtain better results.

Table 3 : Classification results obtained by using proposed method using different feature sets method using different feature sets

Feature set	Cell morphology	Classification accuracy					
		3σ	K-NN				
			K=1	K=2	K=3	K=4	K=5
F1	Flagellated	**93.00**	**95.35**	95.35	95.35	95.35	95.35
	Fimbriated	**84.75**	**93.00**	93.00	93.00	93.00	93.00
F2	Flagellated	91.00	95.35	95.35	93.18	88.63	86.36
	Fimbriated	79.50	93.00	93.00	93.00	93.00	93.00
F3	Flagellated	90.00	95.35	95.35	95.35	95.35	94.72
	Fimbriated	79.00	92.00	92.00	92.00	92.00	92.00
F4	Flagellated	91.50	95.35	95.35	95.35	95.35	95.35
	Fimbriated	80.00	93.00	93.00	93.00	93.00	93.00
F5	Flagellated	92.00	95.35	95.35	93.18	88.63	79.54
	Fimbriated	82.50	92.00	92.00	92.00	92.00	92.00
F6	Flagellated	91.00	95.35	95.35	94.72	94.72	94.72
	Fimbriated	83.00	90.00	90.00	90.00	90.00	90.00
F7	Flagellated	92.50	95.35	95.35	94.72	94.72	94.72
	Fimbriated	84.75	90.00	90.00	90.00	90.00	90.00

The Table 3 summarizes the average classification rates obtained by two different classification techniques, namely, 3σ and K-NN classifier. For testing images, the 3σ classifier has yielded an accuracy of 93% and 84.75% for flagellated and fimbriated cells, where as K-NN classifier has yielded 95.35% and 93%, respectively, in case of K=1(i.e. minimum distance classifier) and feature set F1={x_1, x_3, x_4}. These are the optimal results obtained by using only 3 features and minimum distance classifier (K=1 in K-NN). In general, the performance comparison indicates that the K-NN classifier has good classification ability as compared to 3σ classifier.

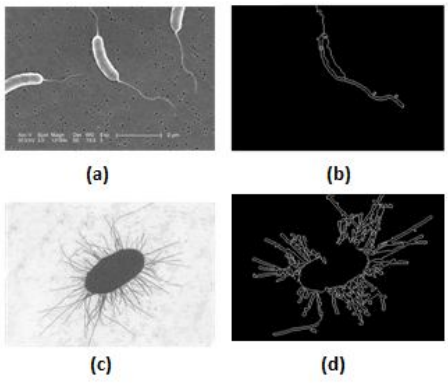

Fig 6: Sample misclassification results (a) original flagellated cell image, (b) Segmented image of image in (a),(c) original fimbriated cell image, (d) Segmented image of image in (c).

CONCLUSION

In this paper, an improved automated cell identification and classification method is proposed. The proposed method comprises the segmentation digital microscopic bacterial cell morphology images and extraction of geometric features of bacterial cell characteristics. The experimental results are compared with the manual results obtained by microbiological expert. The proposed method is computationally less expensive and yet yields comparable classification rates. The 3σ classifier has yielded an accuracy of 93% and 84.75% for flagellated and fimbriated cells, where as K-NN classifier has yielded 95.35% and 93%, respectively, in case of K=1(i.e. minimum distance classifier) and feature set F1={x_1, x_3, x_4}. These are the optimal results obtained by using only 3 features, namely, elongated, eccentricity, and tortousity and minimum distance classifier (K=1 in K-NN). In general, the performance comparison indicates that the K-NN classifier has good classification ability as compared to 3σ classifier.

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