ON-LINE MEASUREMENT OF CELL SIZE DISTRIBUTION AND CONCENTRATION OF YEAST BY IMAGE PROCESSING

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For on-line monitoring of cell size distribution and concentration in a fermentor, a sampling system was developed with a counting algorithm for cells. A digitized microscopic image of yeasts is analyzed based on the Hough transformation, and spherical cells are detected. Then the number of cells and the cell size distribution are calculated. The proposed method was applied to a monitoring of *Saccharomyces cerevisiae* growth and confirmed the method's validity.

Introduction

Cell concentration is one of the most important variables in the analysis and/or operation of bioreactors. To estimate the amount of cells, various methods have been used such as measurement of dry cell weight, packed cell volume and microscopic observation. The microscopic method is substantially reliable, but a large number of cells must be counted in order to obtain statistically representative data. Thus manual counting is often a tedious and time consuming task for researchers.

Contrary to these off-line methods, many investigators have indicated the importance of on-line measurement of cell concentration in bioreactors to control and operate the process. Several on-line methods have been developed, such as the measurement of optical density, electrical impedance³⁾, fluorescence⁵⁾ and so on. These methods, however, cannot determine the concentration accurately in some situations because of the influence of changes in the environment such as those in temperature, culture medium and aeration.

In this study, the role of an operator in counting the cells under a microscope is implemented on a computer. This provides an accurate on-line method of monitoring cell concentration with an automatic sampler. The Hough transform algorithm¹⁾ was applied to on-line counting of the cells. The size of each cell is calculated after detecting particles. The performance of the method was successfully confirmed by standard polymer spheres and was applied to a monitoring of yeast growth.

1. Method

The microscopic image of *Saccharomyces cerevisiae* in cultivation medium is analyzed in this paper. A schematic diagram of the method of particle counting and size calculation is shown in Fig. 1. A detailed description of the procedure is presented in this section.

1.1 Microscopic image

The microscopic image is received by a CCD camera and scanned by a video digitizer, which gives an image of 640×400 dots with gray scale of 256 levels. Figure 2 shows an example of digitized images of the yeast cells at a magnification of 600. As shown in the image, the contrast of the microscopic image is not so sharp and contains some dusts. Each yeast cell is almost spherical and some of them are connected to each other.

1.2 Pre-processing

First, the aspect ratio of the video interface was complemented by Affine transformation. This process is introduced because our video digitizer causes distortion and has no adjustment mechanism. Without this procedure, a circle becomes oval in the digitized image. Affine transformation converts the original point (x, y) to (u, v) ,

$$u = ax + by + c \quad (1)$$

$$v = dx + ey + f \quad (2)$$

where a, b, c, d, e, f are constant coefficients; in our case, $a = 1, e = 0.8$ and the others are zero. The gray scale of each point is, then, linearly interpolated from the nearest four points.

After adjustment of the aspect ratio, a median filter is applied to reduce spike-type noise. The median filter replaces the gray level of each pixel by the median of gray levels in the neighborhood of the pixel. This filter can reduce noise without loss of edge sharpness. The median in a 3×3 neighborhood is used here.

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Fig. 1 Schematic Flow of the Algorithm

Fig. 2 Original Image of Yeast Cells

Next, edges in the image are enhanced by using the Sobel operator. Derivatives based on the Sobel operator masks are:

$$G_x = (z_7 + 2z_8 + z_9) - (z_1 + 2z_2 + z_3) \quad (3)$$

$$G_y = (z_3 + 2z_6 + z_9) - (z_1 + 2z_4 + z_7) \quad (4)$$

where the z 's are the gray levels of the pixels overlapped by the masks at any location in an image (z_1 is top left, z_3 is top right, z_7 is bottom left and z_9 is bottom right in a 3×3 mask). The magnitude of the gradient vector, namely the gray level of edge enhanced image, is defined as follows:

$$g(x, y) = \sqrt{G_x^2 + G_y^2} \quad (5)$$

After that, pixels of the image are partitioned by a single threshold, which is selected automatically⁴⁾ based on the histogram of gray levels of the image.

1.3 Particle detection

After applying the previous processing, linking and other boundary detection procedures are needed. The simplest approach for edge detection is to analyze locally the pattern of the neighborhood pixels and to link them into a short line. Points satisfying the predefined condi-

tions are linked to make an edge. This process is repeated for every location in the image. But this method cannot sometimes recognize a boundary completely because of noise, breaks in the boundary and other effect of discontinuities. The pattern-matching method is one of the other algorithms for particle detection. It needs, however, many patterns for each radius of circles and requires large computation time.

These detection methods are based on analysis of the connected pixels in the image, where as the Hough transform²⁾ uses the accumulated information from many pixels. This method can thus link segments of noisy edges into a continuous line. Because the shape of yeast cells is almost circular, the cells can be distinguished from dusts by employing the Hough transform for a circle.

Assume a point (x_i, y_i) on the edge of a circle of which the center point is (a, b) . The angle θ between these points satisfies

$$\tan \theta = \frac{y_i - b}{x_i - a} \quad (6)$$

The equation can be rewritten as

$$b = (\tan \theta) a + (y_i - x_i \tan \theta) \quad (7)$$

This equation represents a straight line in the parameter space of a and b . Consider a second point (x_j, y_j) ; they can be represented by another line. Thus, every edge point of this circle is represented by a line crossing the center point of the circle. Therefore, detection of this crossing point corresponds to detection of the center point of the circle. In computational practice, this transformation is achieved from subdivision of the parameter space into a so-called accumulator array. An element of the array is incremented when a line occupies the element. After applying this accumulation for every white point, the element having peak count is concluded to be the center point of the circle. The direction θ of the gradient vector at the location of the center of the masks is given by the Sobel operator.

$$\tan \theta = \frac{G_y}{G_x} \quad (8)$$

In practice, the image space is too big in comparison with the cell size; moreover, yeast cells are not complete circles. Hence, the Hough transform produces many intersections in the parameter space. To reduce the complexity, the original image is divided into subdivisions and the Hough transform is applied to a small region on the original image. In this work, 16×16 pixels of regions are treated, and some peak points, not just one, are considered to be candidates for center point of the circle. To decide whether a point is a candidate or not, a threshold of accumulation counter must be predefined. If this threshold value is low, many candidates are produced. If it is high, some particles may be ignored in counting.

Fig. 3 Parameters of Hough Transform

1.4 Size calculation

After obtaining the center point of each circle by the above-mentioned algorithm, the radius of each circle is calculated as follows. From a center point (a, b) , the distance to each non-zero (white) point in the predefined region around the center is calculated. Then, the highest frequency of the calculated distance is considered to occur at the radius of the circle. In practice, a set of pixels at distance R from (a, b) is defined by E .

$$E = \{(x, y) | R = \sqrt{(x-a)^2 + (y-b)^2}\} \quad (9)$$

And the normalized frequency of the edge is defined as follows:

$$S(R) = \frac{\sum_{(x,y) \in E} g(x,y)}{\sum_{(x,y) \in E} 1} \quad (10)$$

where $g(x, y)$ are the pixels of the edge-enhanced image by the Sobel operator. The maximum value of $S(R)$ at various values of R gives the radius of the single yeast cell.

1.5 Particle counting

After obtaining the candidates of the center points and their corresponding radii R , near neighboring points of the true center must be recognized to belong to the same particle. For that purpose, the candidate points are classified in several groups, which together include all the particles. Now imagine two candidate points, and that the distance between them is d . If d is smaller than the corresponding radii of both candidates, it is considered that the point having larger $S(R)$ dominates the other. Applying this method to all candidates, we can determine the center points of the circles.

Fig. 4 Automatic Sampling System

2. Experiments

2.1 Apparatus

A stirred-tank fermentor of $2 \times 10^{-3} \text{ m}^3$ volume is used in this work. The culture of *saccharomyces cerevisiae* is continued at 303.2 K with $3.3 \times 10^{-5} \text{ m}^3/\text{s}$ of air supply. The cultivation medium contained ($[\text{kg}/\text{m}^3]$): glucose, 1; NH_4Cl , 2.5; K_2HPO_4 , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03; CaCl_2 , 0.03; and $3 \times 10^{-3} \text{ kg}$ of yeast extract. Glucose solution ($20 \text{ kg}/\text{m}^3$) was fed at $1.47 \text{ m}^3/\text{s}$, and an ammonia solution is fed so as to control the pH at 5.

An automatic sampler is needed for on-line monitoring of cell concentration by the microscopic method. We therefore developed a sampling system consisting of a liquid feeder and a micro channel for microscopic observation. A schematic diagram of the sampling system is shown in Fig. 4. The cultivation liquid is sampled by a micro-tube pump and fed to the micro channel. After that, the pump is stopped and liquid is held in the channel by closing the electric valves connecting the two sides of the channel. Then the cell image in the channel is observed by a microscope. The channel is flushed by clean water after each sampling to prevent cross-contaminations.

The micro channel is made of silicon film bound in two sheets of glass. The anodic bonding technique is used for the binding of silicon to glass. The depth of this channel, that is, the thickness of the silicon film, is about $200 \mu\text{m}$. The inner volume of the channel is $2.04 \times 10^{-3} \text{ mm}^3$. This micro channel is placed on an electrically controlled X-Y table and several images are obtained from various parts of the channel in a few seconds.

An optical microscope with no phase differential filter is placed for observation of cells in the micro channel. A CCD camera is attached to the microscope and the image is digitized by a personal computer with video digitizing board. The computer also has an ethernet interface and is connected to workstations by a TCP/IP network. A Unix workstation with C language was used for the image-processing calculation. X-window version 11 is utilized to display the original and calculated images.

2.2 Standard particles

To check the validity of the algorithm, the number of standard-size polymer particles is counted. We use standard-size particles of Duke Scientific, having a

Fig. 5 Image of Standard Particles

Fig. 6 Size Distribution of Standard Particles

diameter of $10 \pm 0.3 \mu\text{m}$. Ten sheets of microscopic image with the particles are analyzed by the algorithm. An example of the image is shown in Fig. 5. By manual counting, 190 particles are found. And all particles are counted perfectly by computer, using the method described in the previous section. This result indicates the appropriateness of the algorithm and confirms its high accuracy.

The particle size distribution for these images is also calculated (Fig. 6). This result is accurate enough because the pitch of the digitized image is $0.217 \mu\text{m}/\text{pixel}$.

2.3 Yeast cells

The algorithm described in the previous section has been applied to counting the number of yeast cells in cultivation medium. Before the sampling of the liquid, the yeast was cultivated in the fermentor for about two hours with fed-batch culture operation. This picture has some noise such as dust, and the edge is not always clear because the slide glass has some depth and it is difficult to focus on all the cells in the range of vision.

Figure 2 is an example of the original microscopic image after adjusting its aspect ratio. By human observation, there are 23 yeast cells in this picture. Some of them are connected to each other. After applying median filtering, the edge is enhanced by the Sobel operator, and Fig. 7 is obtained by thresholding the image. Then, the

Fig. 7 Edge-Enhanced Image of Yeast Cells

Fig. 8 Hough Parameter Space

Fig. 9 Detected Particles

parameter space of the Hough transform is calculated, as shown in Fig. 8. In this example, the maximum count in the accumulation array is 50. The threshold value of the accumulation array counter to select a circle is chosen to be 30, and then 47 center-point candidates are found. Moreover, ignoring the candidates within the radius of other circles by calculating each size, the final image, Fig. 9, is obtained. The calculated size distribution of the image is shown in Fig. 10. As a result, 22 particles are detected; that is, one particle cannot be counted. In the original image, the particle that was not counted is of oval shape rather than a circle.

Ten sheets of image are sampled randomly and checked by the counting algorithm. There are 274 cells in total by human observation, but 265 cells are found by computer calculation. By tracing the result more precisely, 14 cells cannot be counted, and 5 cells are mistakenly counted. The total accuracy of cell number is about 3% in this experiment. Considering the error of human

Fig. 10 Size Distribution of Yeasts

counting, this accuracy is satisfactorily high.

The threshold of the accumulation array is set to 30 in this example. The determination of this threshold value is critical to the result of the algorithm. If the value is too high some particles will not be counted, and if it is too low some imaginary circles may be counted. But there is no way to decide this value automatically; it was determined by experience.

3. Discussion

During the sampling of the cultivation medium, some air bubbles were generated due to suction of the tubing pump and breathing of the yeast. Bubbles in the sample medium may be counted as a cell, so a small air trap is installed before the microchannel entrance to remove the air bubbles and it seems to work well. The microchannel is very deep compared with the yeast cell size, and it is difficult to focus on all cells in the channel. So, after introducing the cells into the channel, they are left quietly for about half a minute until most of the cells sink at the bottom of the channel.

The points lying on the circle

$$(x_i - a)^2 + (y_i - b)^2 = c^2 \quad (11)$$

may be detected by using directly the three-parameter Hough transform instead of the two-parameter method which we used. By this method, center points and the circular radii of circles can be detected simultaneously, but this method requires larger memories. Even our 2-D parameter transform requires 256 K bytes of accumula-

tion array, more than a hundred times the memories are required for the 3-D parameter method, so we did not adopt this transform.

Our method can be applied to other cells if their shape is almost spherical. If they are not, another method is needed, such as pattern matching, the generalized Hough transform or others. Whenever the shape is not a complete circle, yeast cells with germination can be successfully counted by our method.

Calculation time for each image depends on the number of non-zero pixels in the image after thresholding. The average of required calculation time was about 30 seconds with AV310C (Data General) workstation for each image. Computer speed will increase in this age of progress, and the required time will become shorter. Some specially designed IC chip was developed for the Hough transform and such a chip will shorten the calculation time drastically.

Conclusion

An on-line monitoring system was developed for yeast cell size distribution and cell concentration. An algorithm for counting spherical cells in cultivation medium is presented. It is based on the Hough transformation and detects only circles among objects of any other shape. Being based on the Hough transform, the method makes high-speed calculation possible with a specialized processor.

An automatic sampling system using a newly developed micro channel was also developed. The performance of the system is evaluated on a yeast culture system and shows good agreement with human observation. This system provides a way of on-line monitoring for dynamic control of cell concentration.

This method can be used to count particles such as bubbles in liquid. For an image including too many particles, dilution may be needed. In combination with dyeing, viable cells can be discriminated from dead cells.

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