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Image Analysis Method for the Rapid Counting of *Saccharomyces cerevisiae* Cells

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An image analysis system which incorporates a microscope, video camera, monitor, and Apple computer and which uses image area to count *Saccharomyces cerevisiae* cells is described and evaluated. Yeast cell suspensions of densities of up to 100 × 10^6 cells per ml can be counted when viewed in the counting chamber of a hemacytometer. The yeast image area measured depends upon the light intensity used to illuminate the yeast cells, the sharpness of the image focused on the monitor, and the grey level selected when scanning the digitized image on the monitor of the Apple computer, all of which can be controlled. The image area also depends upon the yeast strain and medium in which the culture is grown, but it is not affected by the concentration of sugar or ethanol in which the yeast cells are suspended. Yeast growth measured by image analysis can be calibrated to give results similar to those obtained with hemacytometer counting. Yeast cells can be counted in the presence of high cell densities of bacteria by adjusting the grey level at which the digitized image is scanned.

Saccharomyces cerevisiae cell numbers are routinely counted during preparation of starter cultures and for the subsequent fermentations by using a hemacytometer, which is time consuming, or by indirect methods, such as nephelometry or optical density. Sugar and ethanol concentrations vary significantly during fermentation of fruit juice, which affects the yeast cell volume and thus the measurement of turbidity, which must be corrected with either a variable calibration factor or by diluting the cell suspension to a known solute concentration to allow calculation of actual cell densities (4).

The application of image analysis and segmentation to the characterization of surfaces by optical density, size, shape, number, and texture (2) is well established in biology (1, 3, 6). Instrumentation has become increasingly sophisticated in both data acquisition and image processing; however, simple instrumentation should be adequate for image analysis dedicated to counting microbial cells. In this report a relatively inexpensive image analysis system that is constituted of a microscope, video camera, monitor, and Apple computer fitted with a video digitizer and which uses image area to count numbers of yeast cells is described and evaluated.

MATERIALS AND METHODS

Instrumentation and computer hardware. An Olympus transmitted light microscope (BHB) was fitted with a dark-field condenser to obtain contrasting cell images. Video images of cell suspensions contained in the counting chamber of a hemacytometer were obtained with an Ikegami surveillance type camera (CTC 5600) mounted vertically onto the microscope with a standard C-mount adaptor. Video images of cells were displayed on an Ikegami 23-cm (PM-910) black-and-white monitor, and the image was focused. The video display was transferred to the memory of an Apple IIE computer, with a 64K random access memory, monitor, and single disk drive, by using a video digitizing card (Digisector DS-65; The Micro Works, Del Mar, Calif.) placed into slot 5 of the Apple computer.

Computer software. The programming language used was Applesoft BASIC. The image-digitizing program had the main function of scanning a defined frame area (256 by 256 picture elements [pixels]; scan time, 6 s) of the image and recording the cell area for 10 frame scans obtained by manually moving the field of view. The frame area displayed the yeast cells suspended in 7.7 × 10^{-6} and 1.925 × 10^{-6} ml contained in the hemacytometer when the 20× and 40× objectives, respectively, were used. The program could optimize cellular images by altering brightness thresholds (64 grey levels) from the keyboard, define single cell area in pixels per cell, compute sample variables, including dilution factors, and print out the cell density.

Yeast and bacteria cultivation and preparation of suspensions for counting. *S. cerevisiae* strains from the culture collection of this institute were maintained on grape juice agar slants containing 140 g of total sugar per liter solidified with 2% agar. Grape juice medium (GJM) containing Muscat Gordo Blanco grape juice supplemented with 2 g of diammonium hydrogen P_i per liter and a vitamin mixture (5) or MYPG medium containing Oxoid malt extract and yeast extract (3 g/liter), peptone (5 g/liter), and glucose (10 g/liter) were used for growing *S. cerevisiae*. Yeast strains were grown aerobically in GJM and MYPG medium at 25°C in an orbital shaking water bath; yeast cells in the stationary growth phase were harvested by centrifugation, and cell suspensions were loaded into the counting chamber of a Neubauer hemacytometer for visual counting or examination by image analysis. *Leuconostoc oenos* AWRI Lc5b was grown as a static culture at 25°C in Oxoid MRS broth supplemented with 200 ml of clarified tomato juice per liter and 10 g of fructose per liter. Cells were harvested by centrifugation and suspended in water for examination by image analysis. The number of bacteria in suspension was determined as CFU after serial dilution in 0.1% peptone water, spot plated on MRS agar, and incubated at 30°C.

Effect of light intensity and image sharpness on the yeast image area. Yeast strain AWRI 10A harvested from GJM was suspended at 9.0 × 10^6 cells per ml and loaded into a hemacytometer. The light intensity illuminating the yeast cells in the hemacytometer was varied by adjusting the rheostat on the microscope. The mean image area in pixels of 10 frame scans was recorded at each level of light intensity.

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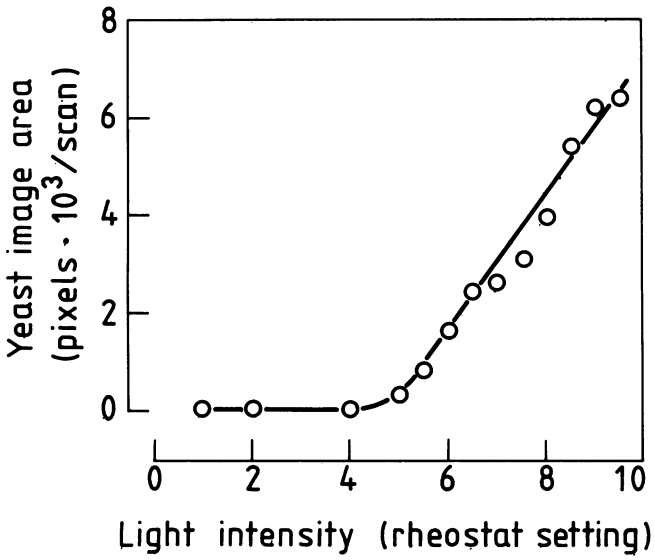

FIG. 1. Image area of 69 yeast cells per scan in response to increased light intensity illuminating yeast cells suspended in water and placed in the counting chamber of a hemacytometer.

A similar yeast suspension of 19.3 × 10^6 cells per ml was loaded into the hemacytometer and illuminated at a constant light intensity. The mean image area was recorded for a range of brightness thresholds selected from the keyboard of the Apple monitor. *L. oenos* suspended at 6 × 10^7 CFU/ml in water was examined for image area under the same conditions as the yeast suspension.

Yeast strain AWRI 10A suspended in water at 40 × 10^6 cells per ml was placed in the hemacytometer, and the image obtained with the video camera was focused on the black-and-white monitor by moving the microscope stage to adjust the depth of focus. The image was transferred to the memory of the Apple computer, the digitized image was scanned, and the image area was calculated. The image was taken out of focus and readjusted 10 times, and the image area was recorded at each setting. Six operators were examined for their ability to focus the image to a reproducible degree of sharpness by comparing the mean image areas obtained. The results were compared with the error in 10 measurements of image area at one focus setting.

Effect of yeast cell density, yeast strain, and growth media on yeast image area. Yeast strain AWRI 10A was suspended in water, with a cell density up to 200 × 10^6 cells per ml. The yeast image area was measured by image analysis for each suspension, and the yeast cell density was determined by counting in a hemacytometer after dilution of high cell densities to ca. 50 × 10^6 cells per ml.

Nine *S. cerevisiae* strains were grown aerobically to the stationary phase in GJM and MYPG media, harvested by centrifugation, and suspended in water, and their cell density was determined from hemacytometer counts. The image area for each cell suspension was recorded, and the mean cell area was calculated.

Effect of glucose and ethanol concentration on the yeast image area. Yeast strain AWRI 10A was suspended at ca. 20 × 10^6 cells per ml in aqueous glucose and ethanol solutions up to 200 g/liter and 200 ml/liter, respectively. The yeast cell density was determined from hemacytometer counts, and the mean image area was recorded at each glucose and ethanol concentration.

Comparison of hemacytometer and image analysis methods for counting yeast cells during fermentation of juice. Duplicate 200 ml portions of GJM contained in 250-ml Erlenmeyer flasks were equilibrated at 25°C in an orbital shaking water bath. The medium was either continuously bubbled with water-saturated air to maintain aerobic conditions or saturated with CO_2 and the flask loosely sealed. The medium was inoculated with yeast strain AWRI 10A grown in GJM to the stationary phase, and the anaerobic and aerobic yeast growth was followed by counting yeast cells in the fermentations in a hemacytometer after dilution to less than 50 × 10^6 cells per ml or by image analysis after dilution to less than 100 × 10^6 cells per ml. Calibration of the image analyzer was made by

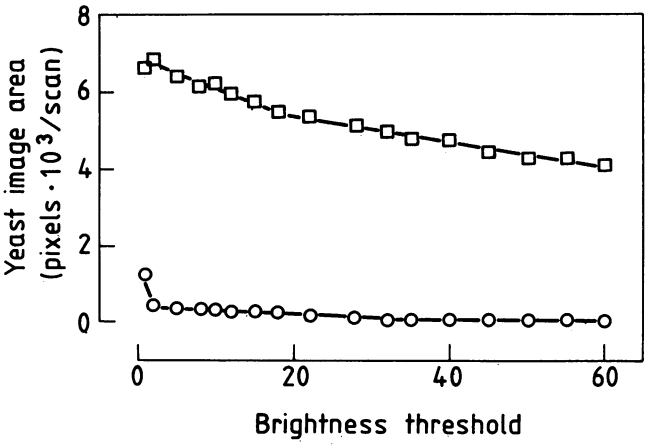

FIG. 2. Image area of 149 *S. cerevisiae* (□) and *L. oenos* (○) cells at different screen brightness thresholds of the monitor.

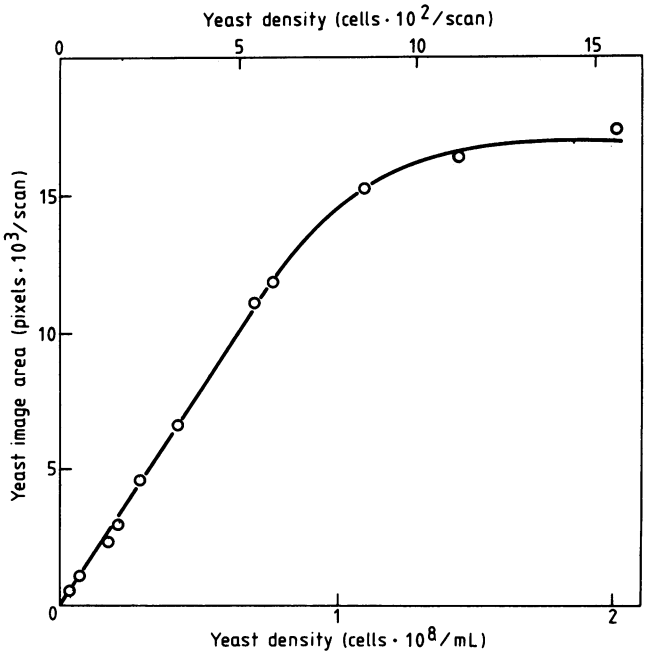

FIG. 3. Image area per scan and number of yeast cells scanned for several cell densities suspended in water and observed in the counting chamber of a hemacytometer.

TABLE 1. Effect of yeast strains and growth medium on the mean image area of yeast cells[a]

S. cerevisiae AWRI strain	MYPG growth medium			GJM		
	No. of cells/ scan	No. of pixels/ scan[b]	No. of pixels/ cell	No. of cells/ scan	No. of pixels/ scan[b]	No. of pixels/ cell
1A	79	2,094	26.5	190	2,415	12.7
2A	72	2,003	27.8	157	2,494	15.9
3A	70	1,808	25.8	117	2,700	23.1
5A	76	2,156	28.4	117	2,812	24.0
8A	58	2,030	35.0	134	3,070	22.9
9A	52	1,673	32.2	151	2,699	17.9
10A	56	1,860	33.2	139	2,931	21.1
11A	51	1,787	35.0	113	3,195	28.3
12A	98	2,471	25.2	146	3,236	22.2

[a] Values were determined from hemacytometer counts and the suspension volume scanned.
[b] Values represent the mean of 10 scans.

using stationary-phase cells similar to those used to inoculate the GJM.

RESULTS AND DISCUSSION

Effect of light intensity and image sharpness on the yeast image area. The yeast image area measured by image analysis increased when the light intensity illuminating yeast cells in the hemacytometer was increased (Fig. 1). There was a critical light intensity below which no image area could be recorded. As the light intensity was increased by changing the rheostat setting on the microscope from 5 to 9, the image area increased linearly from 300 to 6,000 pixels per scan. A rheostat setting of 6 was selected for illuminating yeast cells at subsequent measurements of the image area.

The brightness threshold selected for the monitor screen of the Apple computer affected the image area of the yeast cells (Fig. 2). If the brightness of the screen was reduced by increasing the numerical setting of the threshold, then the image area declined. For an image of 149 cells per scan, the image area measured decreased by 37% as the brightness threshold was varied between 1 and 60. *L. oenos* in suspension at 6×10^7 CFU/ml gave an image area of 1,300 pixels per scan at a screen brightness threshold of 2, decreasing to 20 pixels per scan at a brightness threshold of 30; at lower brightness levels essentially no image area was recorded (Fig. 2). A brightness threshold of 32 was selected when the yeast image area was measured.

TABLE 2. Effect of glucose and ethanol concentration on the image area of yeast cells[a]

Glucose (g/ 100 ml) or ethanol (ml/100 ml) concn	Glucose suspension			Ethanol suspension		
	No. of cells/ scan	No. of pixels/ scan[b]	No. of pixels/ cell	No. of cells/ scan	No. of pixels/ scan[b]	No. of pixels/ cell
0	148	2,961	20.0	148	2,961	20.0
5	145	2,877	19.8	119	2,416	20.3
10	133	3.029	22.8	147	2,489	19.4
15	150	3,280	21.9	116	2,415	20.8
20	127	2,512	19.8	133	2,726	20.5

[a] Values determined were from hemacytometer counts and the suspension volume scanned.
[b] Values represent the mean of 10 scans.

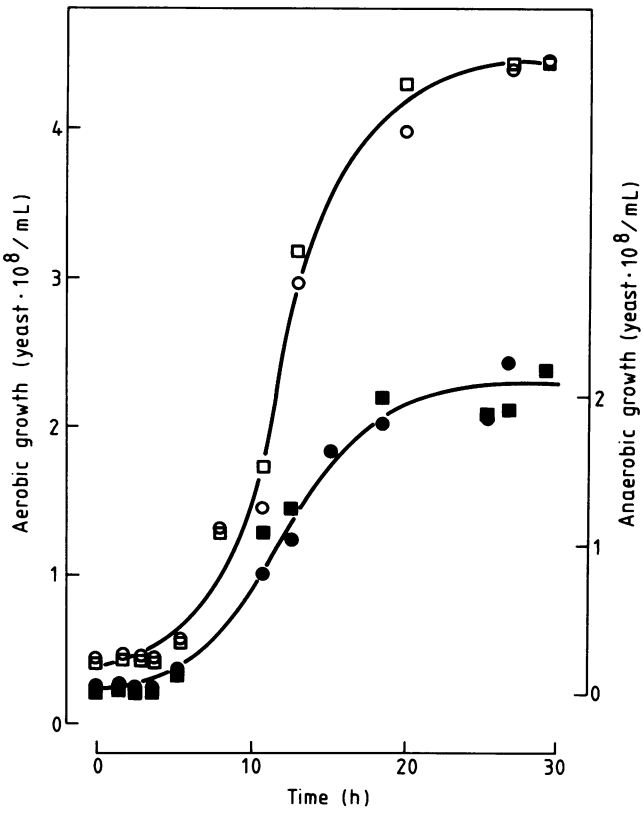

FIG. 4. Aerobic and anaerobic yeast growth in grape juice at 25°C measured by counting in a hemacytometer (□, ■) and by image analysis (○, ●).

The sharpness of the image obtained by the video camera was optimized by focusing the image on a monitor placed between the camera and the computer by adjusting the focal length between the microscope objective lens and the hemacytometer containing the yeast cell suspension. The focal length required to obtain a sharp image on the monitor differed from that required to view the yeast cells through the eyepiece of the microscope. The sharpness of the image affected the image area recorded by the computer when the image was transferred to the computer screen. The coefficient of variance for 10 scans of the same image obtained by six operators after displacing and readjusting the focus of the image between each scan ranged between 1.1 and 2.3%. The coefficient of variance indicates that each operator could readjust the focus reproducibly and that there was good agreement between the data obtained by each operator. A control measurement of 10 scans of the same image with one focus setting gave a coefficient of variance of ca. 1/3 of that obtained by the operators.

Effect of cell density, strain, and growth medium on the yeast image area. The yeast image area obtained at an illuminating light intensity setting of 6 and a brightness threshold of 32 for increasing cell densities is given (Fig. 3). The image area increased linearly with yeast cell density up to 80×10^6 cells/ml, equivalent to 600 yeast cells per scan before crowding of the yeast cells caused the image area to give a saturation response. The sensitivity of the image analysis system with stationary-phase cells of yeast strain AWRI 10A was calculated by linear regression analysis to be 21.7 pixels per cell.

The mean image area of yeast cells varied significantly among yeast strains, and the growth medium affected the size of individual strains (Table 1). The image area was smaller for strains grown in GJM compared with strains grown in MYPG medium.

Effect of glucose and ethanol concentration on image area. The mean image area ± the standard deviation for stationary-phase cells of yeast strain AWRI 10A grown in GJM was 20.9 ± 1.4 and 20.2 ± 0.5 pixels per cell for cells suspended in a range of glucose and ethanol solutions, respectively (Table 2). Neither glucose nor ethanol concentrations, over the range expected during fermentation of juice, affected the image area.

Measurement of yeast growth by image analysis. The increase in numbers of yeast cells during anaerobic and aerobic growth of strain AWRI 10A in GJM measured by counting a hemacytometer and by image analysis is shown (Fig. 4). With image analysis, the illumination of the yeast cells was set at level 6 and the brightness threshold at 32 to obtain a calibration constant for stationary-phase cells and to determine cell numbers during growth. No significant difference between the cell numbers is observed when using hemacytometer counts or image analysis, and the stage of growth, lag or exponential, did not affect the number of cells determined by image analysis with a calibration factor obtained with stationary-phase cells.

Yeast cells in suspension or during growth can be counted by using simple and inexpensive image analysis equipment to relate yeast image area to cell numbers. The yeast cell area varies with strain and growth medium; however, the change in cell volume caused by sugar and ethanol concentrations does not significantly affect the cell area. The apparatus and methods described are being routinely used for determining yeast populations.

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