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Dynamic changes in brewing yeast cells in culture revealed by statistical analyses of yeast morphological data

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The vitality of brewing yeasts has been used to monitor their physiological state during fermentation. To investigate the fermentation process, we used the image processing software, CalMorph, which generates morphological data on yeast mother cells and bud shape, nuclear shape and location, and actin distribution. We found that 248 parameters changed significantly during fermentation. Successive use of principal component analysis (PCA) revealed several important features of yeast, providing insight into the dynamic changes in the yeast population. First, PCA indicated that much of the observed variability in the experiment was summarized in just two components: a change with a peak and a change over time. Second, PCA indicated the independent and important morphological features responsible for dynamic changes: budding ratio, nucleus position, neck position, and actin organization. Thus, the large amount of data provided by imaging analysis can be used to monitor the fermentation processes involved in beer and bioethanol production.

[**Key words:** *Saccharomyces pastorianus*; Bottom-fermenting yeast; CalMorph; Cell morphology; Fermentation; Principal component analysis; Budding profile]

Controlling propagation and fermentation in cellars is an important element in beer fermentation. After raw materials, including malted barley, hops, cereals, adjunct, and water, are converted into wort, brewing yeasts grow and produce ethanol. During fermentation, the production of a well-balanced aroma and the flavor of the final product are at least as important as efficient fermentation and high yield. Quality assurance management, especially of brewing yeasts, is important to maintain their good physiological condition (1).

Several features of brewing yeasts have been used to characterize their physiological states during fermentation. Viability is the traditional method (2). Commonly used viability tests are based on the bright-field stains methylene blue (3) and methylene violet (4) and the fluorescent dye 1-anilino-8-naphthalenesulphonic acid (5). The ability to exclude the dye is dependent on cell viability, so any dead cells are stained. Viability provides information regarding the live population in culture but not about individual living yeast cells.

In contrast, vitality reflects the physiological condition of individual cells during yeast proliferation. Several methods have developed to measure yeast vitality, such as detection of intracellular pH (ICP) (6), measurement of specific oxygen uptake rate (7,8), the acidification power test (9,10), carbon dioxide production (11), vicinal diketone reduction (12), glycogen and trehalose staining (13), and the budding ratio (14).

Of the features analyzed, budding has become of great interest, because the budding ratio correlates with metabolism of polysaccharides and production of bioethanol and other metabolites, such as aroma and flavor compounds (15). Flow cytometry allows fine measurements, including parameters related to the cell cycle (16). More recently, methods combining these three features, viability, cell concentration, and budding ratio, have been developed to simultaneously monitor viability and vitality (17).

To assess quantitatively the morphological features of yeast, we developed an automatic image processing system, CalMorph (18,19). CalMorph directly processes fluorescence micrographs and generates 501-dimensional quantitative data regarding mother cells and bud shape, nuclear shape and location, and actin distribution. Using CalMorph, we can easily, rapidly, and reproducibly generate various quantitative data (20). The program generates reproducible data consistent with those obtained manually. CalMorph can also monitor yeast morphological changes that accompany the budding cycle (20), such as the bud index, and specific morphological features in G1, S/G2 and M cells.

The purpose of this study was to investigate the dynamics of brewing yeast during fermentation. Because the budding profile is a good indicator of cell vitality, we tried to use CalMorph to monitor the physiological state of brewing yeasts during fermentation. However, the hundreds of parameters generated by CalMorph distracted from the objective. Specific parameters could be selected

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DNA, deoxyribonucleic acid; FDR, false discovery rate; FITC-Con A, fluorescein isothiocyanate-Con A; KW test, Kruskal–Wallis test; PCA, principal component analysis; PCs, principal components; Rh-ph, rhodamine-phalloidin.

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in advance based on biological meaning, but this would be arbitrary. The selection of pre-existing parameters was also problematic, because not all informative features were always used. As an alternative approach, we performed successive principal component analyses to extract important time-dependent morphological features. Our results suggest that statistical analyses of morphological data can facilitate yeast management during fermentation.

MATERIALS AND METHODS

Strains, culture conditions, and sample preparation The yeast strain used was the bottom-fermenting yeast *Saccharomyces pastorianus* KBY011 (21). KBY011 was pre-cultured in YPD10 medium containing 1% yeast extract (Becton, Dickinson and Co., USA), 2% peptone (Becton, Dickinson and Co.), and 10% glucose (Nacalai Tesquie, Japan) with shaking at 20°C for 3 days. Harvested cells were diluted to an optical density of $OD_{600} = 0.5$, and then cultured at 20°C for 96 h in 500 mL of YPD10 medium with gentle stirring with a magnetic stir-bar under anaerobic conditions. Samples were collected at 0, 24, 48, 72, and 96 h, and cells harvested by centrifugation. Apparent extracts were analyzed according to a previous reference (22).

Image acquisition and CalMorph analysis Fixation and staining of yeast cells, image acquisition, and CalMorph analysis were performed according to the CalMorph manual (http://scmd.gi.k.u-tokyo.ac.jp/datamine/calmorph/CalMorph-manual.pdf). Briefly, cells (8×10^6/mL) were fixed in 0.1 M potassium phosphate buffer (pH 6.5) containing 3.7% formaldehyde (Wako Pure Chemical Industries, Japan). To obtain fluorescence images of the cell-surface mannoprotein, actin cytoskeleton, and nuclear DNA, cells were stained with fluorescein isothiocyanate-Con A (FITC-Con A, Sigma, USA), rhodamine-phalloidin (Rh-ph, Invitrogen Corp, USA) and 4′,6-diamidino-2-phenylindole (DAPI, Sigma), respectively. CalMorph automatically characterizes each yeast cell by calculating 501 morphological parameters based on data from more than 200 cells. In total, five independent cultures grown under the same condition were analyzed.

Successive principal component analysis To evaluate time-dependent morphological changes, we performed successive principal component analyses (PCA; Fig. 1). First, the 501 morphological parameters were screened to yield 248 parameters that change considerably during fermentation (Fig. 1A). Second, these 248 traits were subjected to PCA to extract the principal components (PCs) explaining the changes (Fig. 1B). Finally, PCA was again applied to the parameters correlating highly with each PC to extract independent parameters (Fig. 1C). All statistical analyses were performed using the 'R' software (http://www.r-project.org/).

In Fig. 1A, the Kruskal–Wallis (KW) test (23) was applied to each parameter to identify large changes. For each parameter, the data consisted by five replications of five time points (25 samples). The false discovery rate (FDR) corresponding to each *P* value of the KW test was estimated by an empirical permutation test of 1000 iterations (24).

In Fig. 1B, PCA was applied to the time-course data of the significantly changed 248 parameters (KW test, FDR = 0.05), after five sets of five replicated sample values were combined across time points, ranked among the combined samples, and summed into one rank-sum value for each time point, as described previously (25). To standardize the rank-sum values among the parameters, the rank-sum values at 0 h were subtracted from the rank-sum values of the other time points. Then, eigenvalues (the variance) and eigenvectors (the rotation) of the rank-sum values were calculated from the covariance matrix for the 248 parameters, the contribution ratio was calculated as the ratio of variance, and the PC score was computed by matrix multiplication between the rank-sum values and the rotation. The PC loadings were computed by multiplication of the rotation (eigenvectors) by the square root of the eigenvalue and dividing by the square root of the variance of the rank-sum. *P*-values for the loadings were computed using a *t* distribution, where *t* process was the same as that used to transform a Pearson's product–moment correlation coefficient into a *t* value (26).

In Fig. 1C, to determine morphological features accompanying PC1 of the first PCA, we selected 50 parameters showing significantly high absolute loadings in PC1 (FDR = 0.15), and a second PCA was performed for the parameters selected using morphological data from 122 replicated wild-types as a null distribution. The 50 parameter values of the 122 replicated wild-type morphological data sets were transformed to a normal distribution using the Box–Cox power transformation, as described previously (26). The eigenvalues (the variance) and the eigenvectors (the rotation) of the 122 transformed wild-type data sets were calculated using the covariance matrix of the 50 parameters. The contribution ratio, PC scores, and loadings were calculated as for the first PCA. The PCs of the second PCA were named in alphabetical order (e.g., PC1, PC2, and PC3 were named PC1a, PC1b, and PC1c, respectively). For PC2, we selected seven parameters; the second PCA was performed in a similar manner.

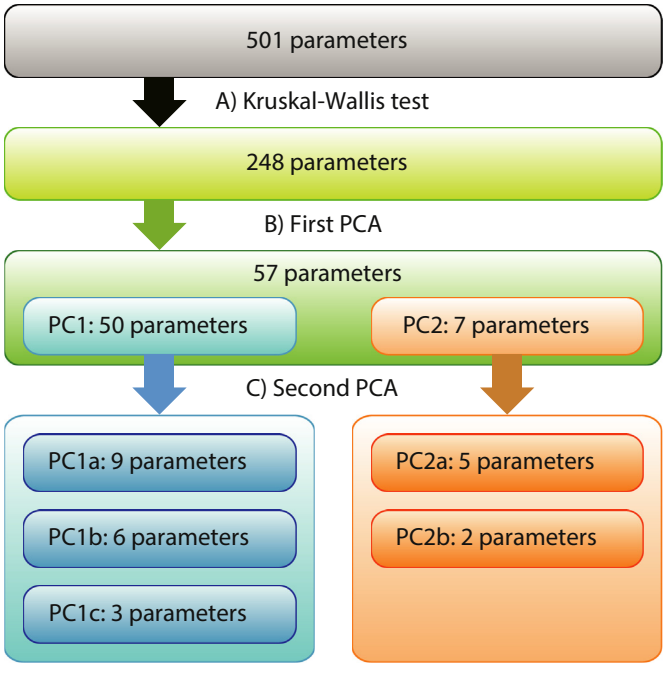

FIG. 1. Schematic flow chart for the successive principal component analysis. Down arrows indicate each step in application of the statistical test or PCA (A–C). The 501 parameters were systematically filtered into semantic fractions by each step. (A) Kruskal–Wallis test was used to select parameters that changed considerably during fermentation and storage. (B) PCA was applied to identify the patterns of the morphological variation. (C) PCA was applied to extract independent morphological features.

RESULTS

Time-dependent morphological changes in fermentation Bottom-fermenting yeast cells were cultured at 20°C with gentle stirring under anaerobic conditions. The changes in cell numbers and apparent extract are shown in Fig. S1. We quantified fermenting yeast cell morphology using 501 morphological parameters. We used image-processing software, CalMorph, after obtaining cell wall, actin, and nuclear DNA images (19). Fermenting yeast cells were sampled at 0, 24, 48, 72, and 96 h, fixed, stained with the fluorescent dyes, FITC-Con A, DAPI and Rh-ph, photographed (at least 200 cells), and quantified using CalMorph. The experiments were replicated five times independently for each time point. As shown in the heat map (Fig. 2), time-dependent changes in the morphological parameters differed in pattern. Of the 501 parameters, 248, 127, and 95 of the 501 differed significantly among the five time points with false discovery rates (FDRs) of 0.05, 0.01, and 0.005, respectively (Fig. 1A).

Key variables in a high-dimensional morphological data set during fermentation PCA is an exploratory multivariate statistical technique for simplifying complex data sets (27). It has been used for analysis of time-dependent changes in gene expression data (28) and dose-dependent changes in morphology data (26). To summarize the morphological dynamics during fermentation, we applied PCA to the morphology data using the morphological measurements as the variables and the different time points as the observations.

The data set contained values for 248 significantly different parameters (FDR = 0.05) collected with five replicates at 0, 24, 48, 72, and 96 h during fermentation. Thus, the matrix to be analyzed had 25 rows of conditions and 248 columns of parameters. Five sets of five replicate sample values were combined across time points, ranked among the combined samples, summed at each time point, and used for the first PCA. Our analysis indicated that we could summarize the data using two variables. The cumulative contri