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BIOTECHNOLOGY METHODS

Automated quantification of budding *Saccharomyces cerevisiae* using a novel image cytometry method

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Abstract The measurements of concentration, viability, and budding percentages of *Saccharomyces cerevisiae* are performed on a routine basis in the brewing and biofuel industries. Generation of these parameters is of great importance in a manufacturing setting, where they can aid in the estimation of product quality, quantity, and fermentation time of the manufacturing process. Specifically, budding percentages can be used to estimate the reproduction rate of yeast populations, which directly correlates with metabolism of polysaccharides and bioethanol production, and can be monitored to maximize production of bioethanol during fermentation. The traditional method involves manual counting using a hemacytometer, but this is time-consuming and prone to human error. In this study, we developed a novel automated method for the quantification of yeast budding percentages using Cellometer image cytometry. The automated method utilizes a dual-fluorescent nucleic acid dye to specifically stain live cells for imaging analysis of unique morphological characteristics of budding yeast. In addition, cell cycle analysis is performed as an alternative method for budding analysis. We were able to show comparable yeast budding percentages between manual and automated counting, as well as cell cycle analysis. The automated image cytometry method is used to analyze and characterize corn mash samples directly from fermenters during standard fermentation. Since concentration, viability, and budding percentages can be obtained simultaneously, the automated method can be integrated into the fermentation quality assurance protocol, which may improve the quality and efficiency of beer and bioethanol production processes.

Keywords Yeast budding · Yeast cell cycle analysis · Image cytometry · Cellometer Vision · Acridine orange · Propidium iodide

Introduction

The brewing and biofuel industries have been utilizing *Saccharomyces cerevisiae*, or baker's yeast, to produce and manufacture their products. Routinely, concentration, viability, and budding percentages of *S. cerevisiae* are measured during production, which can aid in the estimation of the product quality, quantity, and fermentation time of the manufacturing process. Specifically, budding percentages can be used to estimate the reproduction rate of yeast populations, which can indicate and direct the next step in the fermentation process. As replication, or budding, occurs during fermentation, the yeast metabolize polysaccharides in the surrounding media and produce bioethanol, flavor compounds, and carbon dioxide [1]. Typically, the reproduction rate of yeast directly correlates with

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metabolism of polysaccharides and bioethanol production, and can be monitored to maximize production of bioethanol during fermentation [2]. Therefore, the ability to rapidly quantify budding percentages of a yeast population can be used to improve beer and bioethanol production in a standard fermentation process [3].

The traditional method for quantifying budding percentages involves manual counting using light microscopy and a hemacytometer. When a yeast cell reproduces, budding occurs asymmetrically, producing a clearly visible daughter cell that is smaller than the mother cell [4]. Although the morphological characteristics of budding yeast can be easily distinguished using light microscopy, the method is time-consuming and prone to human error, leading to high variability between samples [5]. To improve upon manual counting methods, previous publications have reported the use of fluorescence labeling to analyze and quantify budding yeast [6, 7]. Fluorescence confocal microscopy has been used to visualize calcofluor white-stained "bud scars," which are crater-like formations that are visible after detachment of the bud from the mother cell [7–9]. However, confocal microscopy can only provide the qualification of budding information and cannot be used for quantification of budding percentages of large yeast populations [10]. Flow cytometry has been previously reported to measure the deoxyribonucleic acid (DNA) content during yeast cell cycle, where bud emergence commences in the start of the S phase and continues through G_2/M phase [11]. By employing a nucleic acid-specific stain, it is possible to measure the relative DNA content of each cell, thus determining the replicating yeast population with higher DNA content [6]. However, this method may not be practical in a manufacturing quality assurance setting, as it requires multiple assay steps, lengthy incubation time, and technologically complex systems. In addition, conventional flow cytometry systems are relatively expensive, require highly trained technicians, and most importantly, the fluidic systems are prone to clogging, which poses a significant problem, specifically for biofuel samples containing large debris. Direct quantification of budding yeast using morphological characteristics has been demonstrated using a multispectral flow-based image cytometer [12]. However, due to the fluidic systems, this method can exhibit issues similar to those in conventional flow cytometry.

We have recently demonstrated rapid direct measurement of concentration and viability of yeast populations in corn mash samples [13], as well as a method for the characterization of physiological and metabolic changes in yeast [14] using the Cellometer image cytometry system. The methods can be used in an industrial manufacturing setting to optimize the quality control process, as well as enhance alcohol production efficiency in the research environment. In this work, we developed a novel automated method for the quantification of yeast budding percentages using the image cytometry method. The automated method utilizes a dual-fluorescent nucleic acid dye to specifically stain live cells for imaging analysis of distinguishing morphological characteristics of budding yeast. In order to demonstrate the feasibility of the automated method, a yeast culture was established, and budding percentages were measured at various time points throughout the growth period. The budding percentages were measured under both bright-field and fluorescence-based manual counting methods for comparison. In addition, cell cycle DNA content analysis was also performed at each time point as an alternative method for budding analysis. We were able to show comparable yeast budding percentages between all methods, indicating the feasibility of the developed automated method. To show the capability of the automated method to be utilized in fermentation processes, corn mash samples directly from fermenters at various fermentation time points were analyzed with the automated counting method. Since concentration, viability, and budding percentages can be obtained simultaneously, the automated method can be integrated into the fermentation quality assurance protocol, which may improve the quality and efficiency of beer and bioethanol production processes.

Methods

Yeast sample preparation

The sample preparation procedure is shown in Fig. 1. *Saccharomyces cerevisiae* (*S. cerevisiae)* strain EBY-100 was streaked out onto a petri dish and allowed to culture for 48 h. Next, *S. cerevisiae* were inoculated into 10 ml yeast extract peptone dextrose media (YPD), and incubated in a shaking water bath at 30 °C for 24 h. Following the incubation, 800 µl of the stock liquid culture was diluted into 20 ml of YPD media. Yeast samples were collected at 0.5, 1, 2, 4, 6, 8, 10, 24, and 30 h for budding and cell cycle analysis.

Cellometer instrumentation and disposable counting chamber

The Cellometer Vision image cytometry instrumentation has been described previously [13, 14]. The fluorescence (FL) channels use the fluorescence optics module (FOM) VB-535-402 for FL channel 1 (FL1) and VB-595-502 or VB-660-502 for FL channel 2 (FL2). The combination of microscope objective (10X) and digital camera allows for an optical magnification of 400X and a resolution of $\sim$0.65 µm^2/pixel. The disposable counting chamber and operation method has

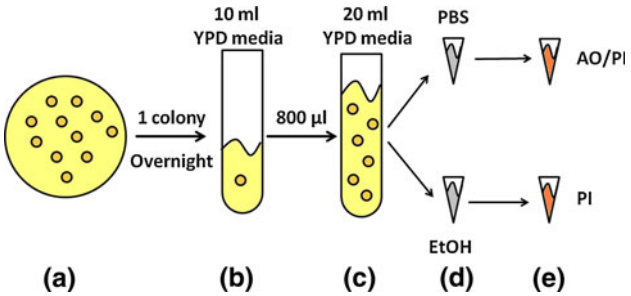

Fig. 1 Schematic of yeast sample preparation and staining procedure. **a** A petri dish of yeast colonies is allowed to culture overnight. **b** One colony is transferred to 10 ml of YPD media and allowed to culture overnight. **c** Next, 800 µl of liquid culture yeast is transferred to 20 ml YPD media, where yeast samples are collected at 0.5, 1, 2, 4, 6, 8, 10, 24, and 30 h time points. **d** Each sample is resuspended in PBS and fixed in ethanol for budding and cell cycle analysis, respectively. **e** Budding is measured using AO/PI staining method and cell cycle is analyzed using PI staining DNA content

been described previously [13, 14]. After imaging analysis of bright-field (BR) and FL images using the Cellometer software, the morphological information (cell size, circularity) and fluorescence intensity of each cell are exported into FCS Express Flow Cytometry (De Novo Software) for yeast budding and cell cycle analysis.

Fluorescent staining for quantification of budding yeast

In order to measure the live yeast budding percentages during a growth period, the cell samples were stained with acridine orange (AO) and propidium iodide (PI) to specifically analyze the AO positive live cells. At each experimental time point, 20 µl of *S. cerevisiae* stock liquid culture was first loaded into a Nexcelom counting chamber to directly determine the concentration of the yeast sample under bright-field cell counting in the image cytometer. Following the concentration measurement, the yeast sample was diluted using 10 mM phosphate buffered saline (PBS) to approximately 3–4 $\times$ 10^7 cells/ml. Next, 10 µl of the yeast sample was mixed with 10 µl of yeast dilution buffer (Nexcelom Bioscience), and then mixed with 20 µl of AO/PI yeast staining solution (Nexcelom Bioscience). The AO/PI-stained yeast sample was vortexed for 5 s to ensure uniform suspension of single cells, and loaded (20 µl) into a counting chamber. Four images were acquired in both BR and FL1 channels at exposure times 15 and 150 ms, respectively. The budding percentages were measured in quadruplicate at 0.5, 1, 2, 4, 6, 8, 10, 24, and 30 h in a normal growth period.

Bright-field and fluorescence manual counting of budding yeast

Manual counting of BR and FL1 images was performed in order to determine the percentage of budding yeast at each time point during the growth period. In this work, a budding yeast cell is defined as a daughter cell at least 1/3 of the size of the mother cell that is in direct contact with the mother cell. The budding percentages were manually determined in quadruplicate at 0.5, 1, 2, 4, 6, 8, 10, 24, and 30 h in a normal growth period. The manual counting results were compared to the automated counting method and cell cycle analysis.

Fluorescence-based automated counting of budding yeast

In order to automatically determine the budding percentages of yeast, Cellometer software was used to export circularity information of counted yeast into FCS Express. The circularity value is defined as 1 for a circle and a value greater than 1 for a non-circular object. Hence, a budding yeast cell would be assigned a circularity value greater than 1, while a non-budding yeast cell would be assigned a circularity value of approximately 1. The Cellometer software was first used to analyze circularity information of the AO-stained yeast in FL1. After exporting to FCS Express, the circularity data for each yeast sample was plotted in a histogram, where the populations with larger circularity values (budding yeast) were gated using linear markers (Fig. 2). The budding percentages were determined in quadruplicate at 0.5, 1, 2, 4, 6, 8, 10, 24, and 30 h in a normal growth period using the automated counting method. The results were compared to the manual counting method and cell cycle analysis.

Cell cycle detection method and analysis

The correlation of budding yeast and yeast cell cycle has been reported in previous publications [12]. In order to perform cell cycle analysis, the Nexcelom Cell Cycle Assay Kit was utilized. At each time point, two ml of the yeast culture were centrifuged at 2,000 rpm (900 $\times$ g) for 10 min. The cell pellet was resuspended and fixed in 800 µl of 95 % ethanol, and incubated on ice for 15 min. Following the incubation, 800 µl of PBS was added prior to centrifugation at 2,000 rpm (900 $\times$ g) for 10 min. The cell pellet was resuspended in 200 µl of cell cycle reagent containing propidium iodide and RNase (Nexcelom Cell Cycle Assay Kit) and incubated at 30 °C for 40 min. Following the incubation, the cells were washed and resuspended in PBS at a final concentration of approximately 5–7 $\times$ 10^7 cells/ml. Then 20 µl of the PI-stained yeast was loaded into a counting chamber and analyzed using the FL2 channel (VB-595–502). The image cytometer captured eight fluorescent images at an exposure of 4,000 ms. The yeast cell cycle was analyzed in quadruplicate at 0.5, 1, 2, 4, 6, 8, 10, 24, and 30 h in a normal growth period, and the

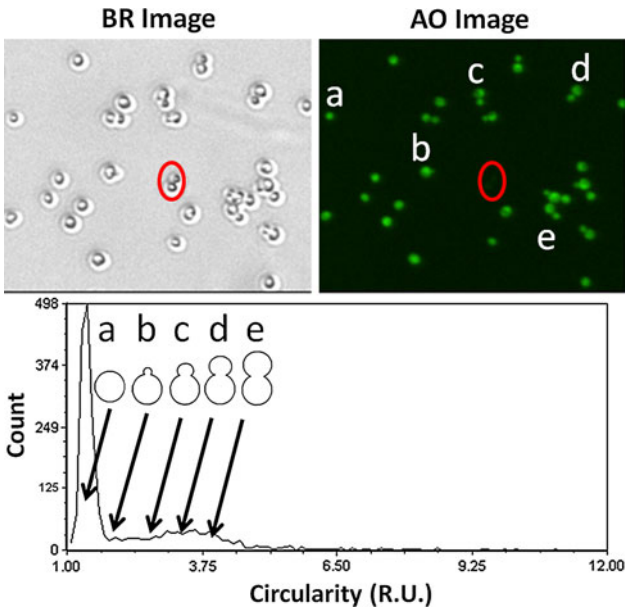

Fig. 2 Yeast budding automated analysis method showing image cytometry captured bright-field and fluorescent image (AO/PI) of yeast. The AO fluorescent image shows clear fluorescence of single and budding yeast particles, which are analyzed by the software to generate circularity data. The circularity data is plotted in a histogram to show the budding yeast population. **a** Single yeast particle has the highest population, and as the yeast particle begins to bud, the circularity value increases from **b** to **e**. Each budding shape example is shown in the AO fluorescent image. Note the *red circle* shows some yeast-like particles are counted in the bright-field images, but they are non-fluorescent, which indicates the particles are non-nucleated. This observation can potentially introduce counting error in the bright-field images

fluorescence intensity data was exported to FCS Express for population analysis.

Under FCS Express, the fluorescence intensities of each yeast sample were plotted in a histogram that displayed a normal cell cycle curve. The yeast populations with greater fluorescence intensity than G_0/G_1 at each time point were gated to measure the percentage of yeast with higher DNA content, which could be used to indicate yeast replication. The results were compared to the automated and manual counting methods.

Direct budding percentage measurement of fermentation corn mash samples

The fermentation corn mash samples were provided by Lincolnway Energy (Nevada, IA). Five samples were collected directly from fermenters at 1.5, 8, 23, 39, and 54 h into 500-ml Nalgene® bottles. Each bottle of collected sample was diluted 1:10 in H_2O in a 50-ml conical tube prior to AO/PI staining. The image cytometry method was used to simultaneously measure the budding percentage, concentration, and viability following the AO/PI staining protocol. The AO/PI detection for viability utilized the FOM VB-535-402 and VB-660-502, respectively. The experiment was performed in quadruplicate.

Results

Manual budding measurement

In order to validate the automated method for quantifying yeast budding, a traditional manual counting method was employed for comparison. Bright-field and fluorescent images of AO/PI-stained yeast are shown in Fig. 3. The manually counted budding percentages at each time point are 26.3, 30.2, 71.9, 57.5, 68.4, 53.5, 37.8, 25.3, and 25.4 %, for bright-field and 24.3, 29.8, 69.9, 58.3, 63.0, 52.2, 37.3, 23.3, and 23.4 %, for fluorescence (Fig. 5). The results between both bright-field and fluorescence manual counting are highly comparable.

Automated budding measurement

The image cytometry-based yeast budding quantification results were compared directly to standard manual counting. Fluorescent images of AO/PI-stained yeast are shown in Fig. 3. The fluorescent images were analyzed to generate the circularity data, which was plotted in FCS Express (Fig. 3). The population with obviously higher circularity values (budding yeast) was measured. The budding percentages measured from the histogram at each time point are 26.1, 28.7, 71.5, 61.1, 61.4, 49.3, 33.9, 22.0, and 22.1 % (Fig. 5). The data were consistent with manual counting results in bright-field and fluorescence, demonstrating the accuracy of automated budding measurement method compared to the standard manual method (2–10 % difference).

Cell cycle analysis

Cell cycle analysis has traditionally been performed to determine the deoxyribonucleic acid (DNA) content of yeast during replication, which poses as an alternative in determining actively replicating or budding yeast [6]. Cell cycle analysis of yeast was performed to further validate the automated method for quantifying yeast budding. Fluorescent images of PI-stained yeast at various stages of the yeast cell cycle are shown in Fig. 4. Exported fluorescence intensity data was used to plot the cell cycle histograms of each yeast population (Fig. 4). Budding percentages were obtained by gating populations with fluorescence intensities greater than G_0/G_1. The measured budding percentages are 27.9, 28.8, 68.3, 64.1, 58.0, 49.7, 33.1, 20.1, and 19.0 % (Fig. 5), which are consistent with those determined by the manual and automated counting methods.

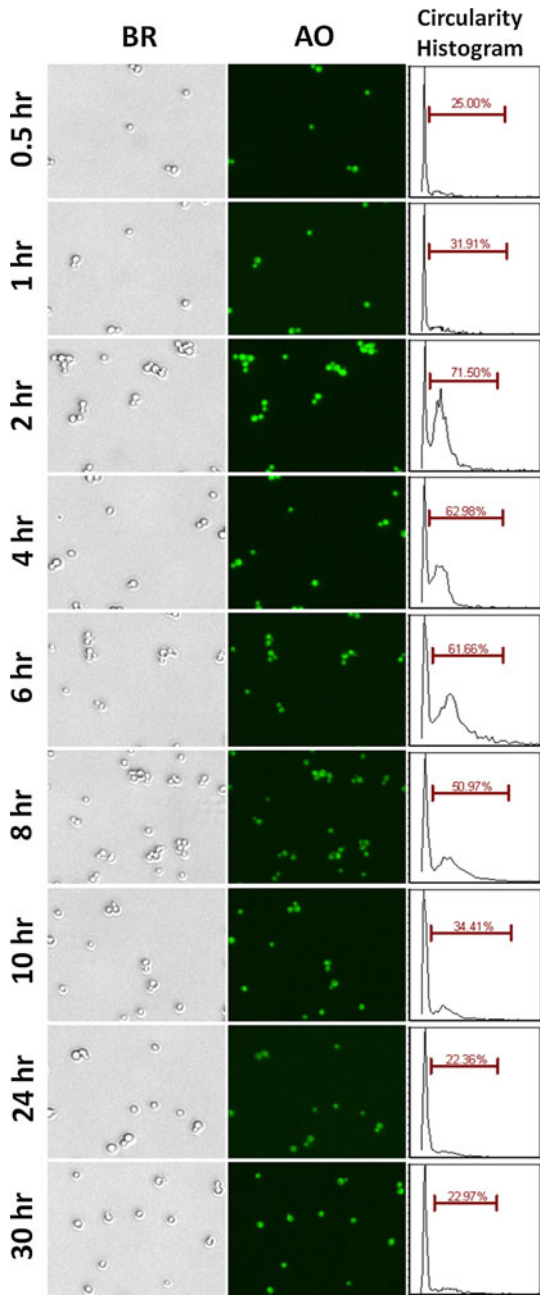

Fig. 3 Manual and automated budding analysis of growth period. Manual counting of bright-field and AO fluorescent images are performed at each time point. Specifically, total yeast and budding particles are counted in the images to generate budding percentages. The automated budding analysis is also performed at each time point, where the budding populations are gated to measure percentages

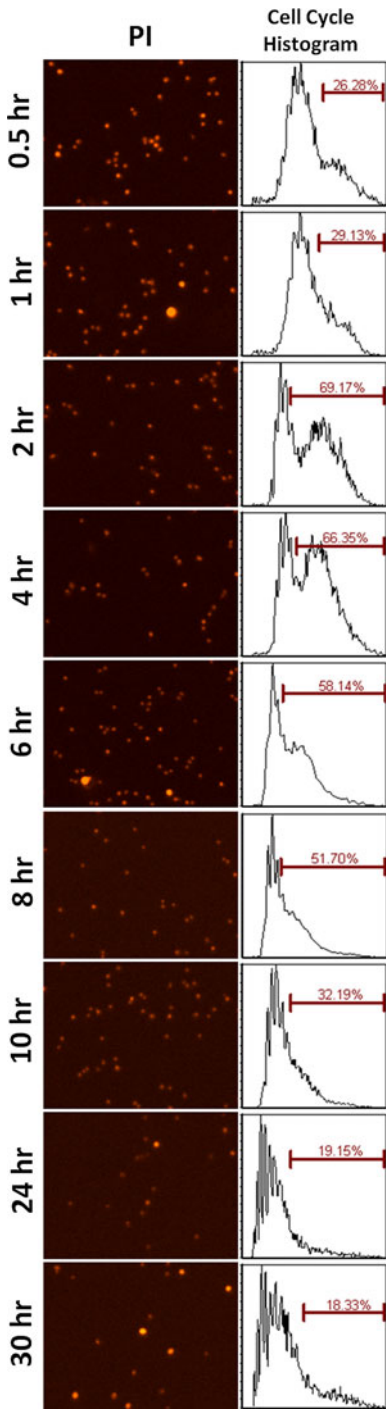

Fig. 4 Fluorescence intensity histogram of PI for cell cycle analysis. Yeast samples were collected at different times (from 0.5 to 30 h) during the cell cycle and stained for the DNA content. Fluorescence intensities are measured by gating cell populations with DNA content greater than G_0/G_1, which can be correlated to active replicating yeast cells

Simultaneous budding percentage, concentration, and viability measurement of fermentation samples

In order to show the capability of the automated method for measuring industrial fermentation samples, corn mash samples were directly collected from fermenters for analysis of budding, concentration, and viability simultaneously. Figure 6a showed the viability and budding percentages of yeast in corn mash at each time point. The viability of yeast increased from 68.07, 77.00, to 85.25 % in the first 23 h of

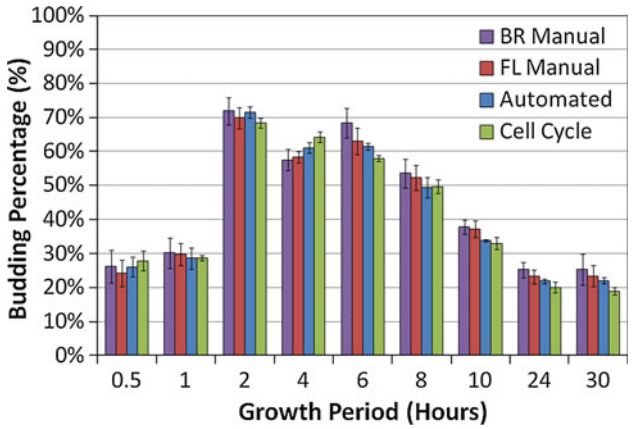

Fig. 5 Comparison of budding percentages measured by manual and automated cell counting, as well as cell cycle DNA content analysis (Bar graph of BR Manual to Cell Cycle are shown from left to right). The measured budding percentages are highly comparable between each method, showing an initial doubling of budding percentages at approximately 2 h, which correspond to the doubling of *S. cerevisiae*. As the nutrients deplete, the budding percentages decrease due to lack of yeast activity in nutrient deprived media. It seems that there is a basal level of active budding yeast of ~20 % even at 30 h of incubation

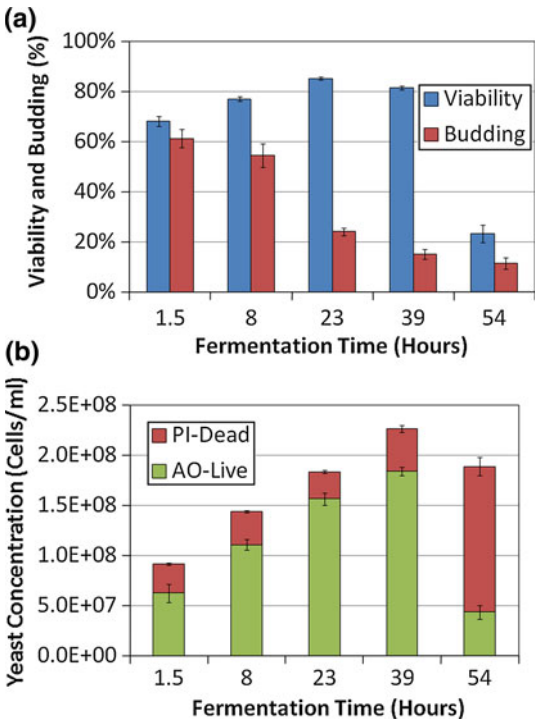

Fig. 6 Correlation of budding percentages, concentration and viability of yeast fermentation samples. **a** Samples showed steady increase in viability (*left*) as fermentation time increased until 54 h, where viability decreased from ~80 to 20 %. Budding percentages (*right*) showed steady decrease from ~60 to 10 % throughout the entire fermentation process. **b** Samples showed steady increase in live cell concentration, which ultimately reduced at 54 h

fermentation, and then decreased from 81.48 to 23.13 % in the next 31 h. In contrast, the budding percentages showed a steady decrease in the first 8 h from 61.29 to 54.50 %, and then abruptly reduced to 24.06 % at 23 h. In the later fermentation hours, the budding percentages further reduced to 15.09 and 11.51 %. The live and dead cell concentrations are shown in Fig. 6b. The live cell concentration increased steadily from 6.25×10^7 to 1.84×10^8 cells/ml in the first 39 h, and then reduced abruptly to 4.37×10^7 cells/ml at 54 h. In correlation, the dead cell concentration remained consistent in the first 39 h from 2.91×10^7 to 4.21×10^7 cells/ml, and then increased to 1.45×10^8 cells/ml. This data showed a clear correlation between yeast budding and its viability.

Discussion

The ability to rapidly quantify yeast budding is of great importance to the biofuel and brewing industries, who commonly acquire yeast budding information to determine the optimal procedure in the manufacturing process. The current method of quantifying yeast budding relies mainly on manual counting using a hemacytometer, which is time-consuming and prone to human error. By using the combination of image cytometry and a fluorescent viability dual-staining reagent, we were able to develop a novel automated detection method for the simultaneous quantification of budding percentages, concentration and viability in a yeast population. In order to validate this automated method, the results obtained from traditional manual counting in bright-field and fluorescent images, as well as cell cycle analysis, were compared to those obtained from the image cytometry method. Budding percentages measured at each time point during the growth period were consistent between manually counting in bright-field and fluorescent images, the automated counting method, as well as DNA content analysis.

The results obtained from all four methods were consistent throughout the entire growth period. The measured budding percentages showed that, in the first 2 h, the budding percentages increased by greater than two times, which corresponded closely to the doubling rate of *S. cerevisiae* [15]. After 2 h, the budding percentages gradually decreased to the basal budding level due to the deprivation of nutrients. Although the four methods used to measure yeast budding showed highly comparable results, the percentages determined by bright-field manual counting were slightly higher than the other three methods for seven of the nine experimental time points. We hypothesized that this may be due to the difficulty of distinguishing individual yeast in clusters of cells in bright-field images. In contrast, fluorescent

images display visually clearer separation between individual yeast, given that optimized fluorescence exposure time is utilized. The difficulty in distinguishing individual yeast in cell clusters in bright-field images can lead to an erroneously low total cell count, thus generating higher budding percentages compared to the other fluorescence-based methods.

The automated budding quantification method depends mainly on fluorescent imaging analysis. Fluorescent imaging utilizes a dual fluorescent nucleic acid dye (AO/PI) to fluorescently stain individual live yeast particles, which has been described previously [13, 14, 16, 17]. Because the viability of each yeast sample was greater than 99 %, only the AO images were analyzed for live budding analysis. Fluorescence imaging of AO positive cells increases contrast, which allows image recognition analysis of individual yeast particles. Since AO nucleus staining can be localized to individual cells, fluorescence imaging can exhibit clearer separation between clusters of cells as compared to bright-field imaging. In addition, nonspecific debris is not stained with AO/PI and is excluded from imaging analysis.

It was necessary to perform cell cycle analysis as an alternative method for measuring budding in actively replicating yeast population and to validate the developed automated method. Manual and automated counting are both image-based methods that are dependent on the morphology of the cell. In contrast, cell cycle analysis analyzes the relative DNA content in the cell population and is independent of yeast morphology. The cell cycle analysis method utilizes a PI/RNase staining solution, which allows specific DNA content measurement. As DNA is replicated in S phase, cell populations in S, G_2/M phase have higher DNA content than those in G_0/G_1 phase, and emit a correspondingly stronger fluorescence signal. It has been previously reported that bud emergence occurs during S phase, and the yeast in S and G_2/M phase are considered to be undergoing budding [4, 6]. Although cell cycle analysis can be used as an alternative method to measure budding percentages, the long incubation time and multiple assay steps may not be feasible for an industrial manufacturing setting.

In addition to the automated budding quantification, Cellometer image cytometry can be used to simultaneously measure concentration and viability of a yeast population. In the results obtained from fermentation samples, one can observe a clear correlation between budding, concentration, and viability. The concentration of yeast increased due to high budding percentage, but reduced in growth rate (from 7.42×10^6 to 1.73×10^6 cells/ml h) as the budding percentage decreased. Since budding activity, or yeast replication, was reduced, new viable yeast were not produced, thus increasing dead cell concentration and decreasing viability. Please note that bioethanol content is also a factor that reduces cell viability during fermentation, as shown in a previous publication [13].

The development of a fast, accurate, and simple yeast analysis method can improve the current industry standard method, which relies mainly on manual counting using a hemacytometer. With the combination of these three parameters (concentration, viability, budding percentage), the fluorescence-based image cytometry method can be used to easily monitor yeast population characteristics during fermentation, which can allow researchers in the biofuel or brewing industry to improve their fermentation process, as well as improve the efficiency of quality assurance protocols. Future work may also involve supplementing the detection process with a yeast vitality parameter to complete the characterization of yeast during fermentation. We have demonstrated the capability of the image cytometry method for quantifying yeast budding via morphology and DNA content. This automated method can reduce the time required to obtain yeast characteristics in an industry setting, which is of great importance for the optimization of the fermentation process.

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Conflict of interest The authors declare a competing financial interest in the work described in this manuscript that is aimed at instrument performance reporting for Nexcelom Bioscience, LLC. The performance of the instrumentation and reagents has been compared with standard approaches currently used in the fermentation industry.

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