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ORIGINAL PAPER

Direct concentration and viability measurement of yeast in corn mash using a novel imaging cytometry method

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Abstract Worldwide awareness of fossil-fuel depletion and global warming has been increasing over the last 30 years. Numerous countries, including the USA and Brazil, have introduced large-scale industrial fermentation facilities for bioethanol, biobutanol, or biodiesel production. Most of these biofuel facilities perform fermentation using standard baker's yeasts that ferment sugar present in corn mash, sugar cane, or other glucose media. In research and development in the biofuel industry, selection of yeast strains (for higher ethanol tolerance) and fermentation conditions (yeast concentration, temperature, pH, nutrients, etc.) can be studied to optimize fermentation performance. Yeast viability measurement is needed to identify higher ethanol-tolerant yeast strains, which may prolong the fermentation cycle and increase biofuel output. In addition, yeast concentration may be optimized to improve fermentation performance. Therefore, it is important to develop a simple method for concentration and viability measurement of fermenting yeast. In this work, we demonstrate an imaging cytometry method for concentration and viability measurements of yeast in corn mash directly from operating fermenters. It employs an automated cell counter, a dilution buffer, and staining solution from Nexcelom Bioscience to perform enumeration. The proposed method enables specific fluorescence detection of viable and nonviable yeasts, which can generate precise results for concentration and viability of yeast in corn mash. This method can provide an essential tool for research and development in the biofuel industry and may be incorporated into manufacturing to monitor yeast concentration and viability efficiently during the fermentation process.

Keywords Bioethanol · Corn mash · Fermentation · Yeast viability · Fluorescence detection · Cellometer® · Imaging cytometry

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Introduction

Biofuel production has dramatically increased over the last 20–30 years [2, 4, 30]. With the continuing depletion of fossil fuels and increasing concern regarding global warming, large-scale industrial fermentation facilities have been introduced in Brazil, the USA, and many European countries to establish a renewable energy source [4, 8]. Currently, the most common biofuel process is bioethanol production, which utilizes baker's yeast, *Saccharomyces cerevisiae*, for fermentation of sugar cane, polysaccharides, waste water, and corn maize (wet-milled or dry-ground) [30]. The high ethanol tolerance, final ethanol concentration, glucose conversion rate, and historical robustness of industrial fermentation make yeasts the ideal organism for bioethanol production [3, 8]. Research and development for enhancing bioethanol production may involve studying the effects of fermentation parameters such as yeast strains and fermentation conditions (yeast concentration, viability, temperature, pH, nutrients, etc.). Specifically, monitoring yeast concentration and viability is important for quality control of the fermentation process in both research and manufacturing [6]. Selection of a higher ethanol-tolerant yeast strain may prolong the fermentation cycle, which has the potential to increase bioethanol yield. To identify an

optimal yeast strain, the viability of various yeast strains under different fermentation conditions can be explored [3, 10, 18]. Therefore, developing a simple counting method for measuring yeast concentration and viability could be essential for optimizing bioethanol production.

There are several methods for concentration and viability measurements of pure yeast samples, such as light and fluorescence microscopy using a hemocytometer, flow cytometry, and fluorescence microscopy [23]. The hemocytometer is low cost and simple to use, but the process is tedious and prone to human error that may lead to large inconsistency from count to count [26]. In contrast, flow cytometry automates data acquisition, but requires experienced technicians for instrument operation and thorough cleaning procedures to prevent cross-contamination between samples, and is expensive to maintain [16]. The two methods described above can provide relatively accurate concentration and viability measurements on pure yeast samples [11, 32].

Counting yeast in corn mash (YCM) relies mainly on manual methods using a hemocytometer, which may be due to the fact that corn mash debris can easily obstruct a flow-based system, and also generates nonspecific fluorescent signals that can prevent consistent results for flow cytometry [27]. Methylene blue is the standard industry method for staining nonviable yeasts in manual counting, but it is often difficult for technicians to identify and separate nonviable yeasts from corn mash debris, thus reducing the consistency of concentration and viability measurements [1, 5, 6, 28, 29]. The method can be time consuming depending on the density of corn mash, the counting results can be technician dependent, and it may be difficult to automate [5, 6, 17, 27].

Fluorescence microscopy detection of yeast has been studied extensively in the past [11, 15, 22, 32], but the majority of reported work involved use of pure yeast samples. To understand how the fluorescence of yeast behaves in a corn mash medium, initial experiments were performed by staining YCM provided by Lincolnway Energy with various commercially available dyes such as SYTO 9 (Invitrogen), carboxyfluoroscein diacetate (CFDA, Invitrogen), calcein AM (Invitrogen), calcofluor (Invitrogen), acridine orange (Biotium), propidium iodide (Biotium), ethidium bromide (Biotium), and 4',6-diamidino-2-phenylindole (DAPI) (Biotium) (Fig. 1). The staining procedures for each dye were provided by Invitrogen and Biotium. The fluorescence signals for each dye resulted in either weak target cell signals (Fig. 1b–e, h) or large nonspecific staining of corn mash debris (Fig. 1a, f, g), which increase the difficulty in differentiating between viable and nonviable yeasts. Therefore, it would be beneficial to develop a simple fluorescence method that can automatically measure concentration and viability using yeast directly from fermenters.

Recently, a novel imaging cytometry system (Cellometer®) has been developed by Nexcelom Bioscience (Lawrence, MA), which allows rapid measurement of cell concentration using disposable counting chambers that require only 20-µl samples [25]. Utilizing combined bright-field (BR) and fluorescence (FL) imaging, the system automates cell image acquisition and processing using a novel counting algorithm for measurement of cell population and viability on a variety of cell types. Applications such as quantification of green fluorescent protein (GFP) transfection, viability using trypan blue or propidium iodide, and enumerating white blood cells (i.e., nucleated cells) in whole blood have been previously reported. More importantly, the method has been shown to produce consistent concentration and viability measurements of pure yeast for quality-control purposes of industrial fermentation and yeast propagation in biofuel and alcoholic beverage industries [20].

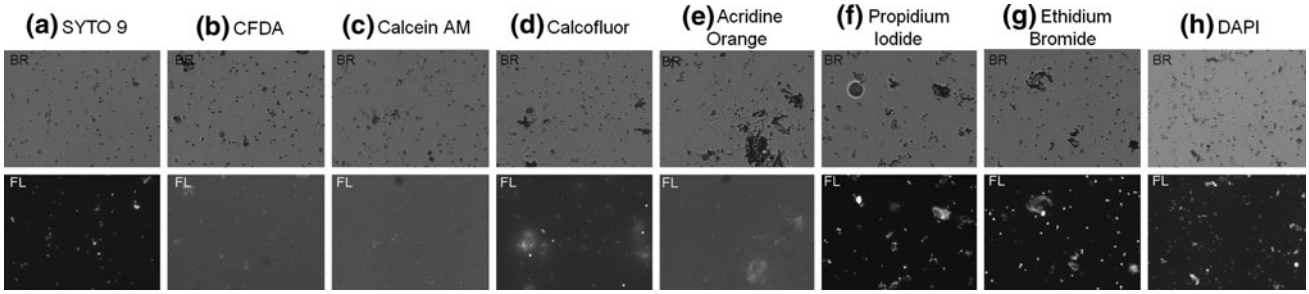

Fig. 1 Initial observation of yeasts in corn mash (YCM) using eight fluorescent stains: **a** SYTO 9, **b** CFDA, **c** calcein AM, **d** calcofluor, **e** acridine orange, **f** propidium iodide, **g** ethidium bromide, and **h** DAPI. The *top* and *bottom rows* show bright-field (BR) and fluorescence (FL) images, respectively, of YCM stained with each dye. The BR images show the complexity of the YCM samples, where the corn mash debris may be mistakenly counted as nonviable cells when using methylene blue staining. The YCM was diluted in phosphate-buffered saline (PBS), and the resulting fluorescence images show nonspecific staining of corn mash (**a**, **f**, **g**) and weak fluorescence signals of viable cells (**b**–**e**, **h**). Weak fluorescence signals are problematic for automated imaging analysis when performing the enumeration process due to indistinguishable yeast cells. Large nonspecific staining of corn mash generates issues for the software when differentiating yeast cells from debris

In this work, we demonstrate an imaging cytometry method employing the Cellometer system for determining concentration and viability of YCM from operating fermenters. Utilizing a yeast dilution buffer and yeast staining solution (nucleic acid dye) from Nexcelom Bioscience, the viable and nonviable yeast are selectively labeled while nonspecific fluorescent signals from corn mash are eliminated. This represents a valuable method for research in bioethanol production improvement, which can also be used to perform effective yeast quality control in the standard fermentation process.

Methods

Yeast sample preparation

The YCM samples were provided by Lincolnway Energy (Nevada, IA). Seven YCM samples were collected directly from fermenters at 2.65, 8, 10, 25, 39, 45, and 55 h (samples 1–7) into a 500-ml Nalgene® bottle. The average pH value was 4.45 ± 0.18, as measured using a Shimadzu high-performance liquid chromatography (HPLC) system (Shimadzu, Japan). Each bottle of collected sample was used to measure concentration and viability using the imaging cytometry method and hemocytometer.

Instrumentation and disposable counting chamber

The instrumentation utilizes an epifluorescence optical setup for one bright-field and two fluorescence channels to perform image-based cytometric analysis. The bright-field and fluorescence channels use monochromatic light sources with excitation and emission filters. For FL channel 1 (FL1), the excitation and emission filter are set to 475 and 535 nm, respectively. For FL channel 2 (FL2), the excitation and emission filter are set to 540 and 670 nm, respectively. The system has a motorized assembly for automatic switching between the two channels and acquisition of images at different wavelengths. This two-color imaging system is used to measure viable and nonviable cell concentration, and automatically calculates the viability for each sample. The disposable counting chamber holds precisely 20 µl of sample. Four separate areas are imaged and analyzed on the imaging platform, where the target cells are identified and counted by the software. The software identifies and counts the viable and nonviable cells in FL1 and FL2, respectively. The concentration is automatically calculated by dividing the total cell count (FL1 + FL2) by the imaged volume, and the viability is calculated by dividing the FL1 cell count by the total cell count.

Buffer selection for concentration and viability measurement

Buffered Reagent Kit (BRK) dilution buffer is a high-ionic-strength reagent from Nexcelom Bioscience (Lawrence, MA), which is used to dilute yeast samples from fermenters, enhance target yeast fluorescence intensity, and eliminate nonspecific staining of corn mash. Phosphate-buffered saline (PBS) at 0.1 M and cell-culture-grade H_2O were purchased from Sigma–Aldrich (St. Louis, MO). Fluorescence intensities from YCM diluted in the BRK dilution buffer, PBS, and cell-culture-grade H_2O were compared to select the optimal buffer.

One milliliter of YCM (sample 2) from the 500-ml Nalgene bottles was diluted 1:20 by volume with the BRK dilution buffer, PBS, and H_2O (19 ml) in a 50-ml conical tube. Since the YCM was a thick slurry solution, the 500-ml Nalgene bottle was shaken well before pipetting. After premixing each buffer with YCM, the conical tube was mixed by inverting ten times. The diluted YCM (10 µl) was then mixed with 10 µl yeast staining solution [acridine orange, propidium iodide mixture (AOPI)], and allowed to incubate for 2–3 min. Acridine orange (AO) is a cationic membrane-permeable dye that, when used alone, labels all cells with green fluorescence. Propidium iodide (PI) is an intact-membrane-exclusion dye that readily penetrates membrane-compromised (nonviable) cells, producing orange fluorescence [7, 13, 14, 24, 31]. Since a significant portion of the AO emission spectrum overlaps with the excitation of PI and both dyes are co-localized in the nucleus of nonviable cells, fluorescence resonance energy transfer (FRET) occurs when AO emission energy is transferred to PI, so that nonviable cells do not fluoresce green [9, 12, 19, 21]. The combination of AOPI staining enables a live/dead cell identification assay to specifically label viable and nonviable cells. The stained diluted YCM (20 µl) was then pipetted into a cell counting chamber and allowed to settle into a monolayer for 1 min. Each diluted YCM was then counted and analyzed using the imaging cytometry system in triplicate for concentration and viability measurements.

After the selection of optimal buffer (BRK buffer), viability measurements were made over a period of 5 h to ensure that the yeast viability was stable when diluted in the BRK buffer. Next, the concentration and viability of YCM samples 1–7 were measured following the same procedure described above. Each YCM sample was analyzed in triplicate, and the results were compared with those of the hemocytometer method.

Manual counting method using Neubauer hemocytometer

YCM samples 1–7 were diluted 1:40 with the BRK buffer and stained with methylene blue for manual counting at

final concentration of 20 ppm. Each YCM sample was counted twice using a standard Neubauer hemocytometer. The concentration and viability results were compared with automated counting by the software.

Results and discussion

Buffer selection

The BRK buffer was compared with PBS and cell-culture-grade H_2O to obtain the highest yeast fluorescence signal-to-background intensity (S/B) ratio. Fig. 2a, b shows the S/B ratio measurements for viable and nonviable yeast diluted in each buffer. The S/B ratios for viable yeasts in YCM sample 2 were approximately 8.3, 1.1, and 3.1 for BRK buffer, PBS, and H_2O, respectively (Fig. 2a). For nonviable yeast, the S/B ratios were approximately 40.5, 17.4, and 33.0 for BRK buffer, PBS, and H_2O, respectively (Fig. 2b). The fluorescence intensity of YCM sample 2 diluted in BRK buffer showed the highest S/B ratio for both viable and nonviable yeast. PBS and H_2O were deemed inappropriate as staining buffers, since they exhibited similar fluorescence results as in Fig. 1, where viable yeasts did not have the required fluorescence intensity to be accurately counted by the software. By diluting YCM samples in BRK buffer, the fluorescence intensity of yeasts was enhanced compared with in PBS and H_2O (Fig. 2c, d). In addition, nonspecific fluorescence of corn mash was reduced for both AO and PI in the BRK buffer (Fig. 2c, d). Therefore, the BRK dilution buffer was selected to perform the concentration and viability measurements for the seven YCM samples. To confirm that the BRK buffer did not affect viability, an experiment was performed where yeasts were incubated in the buffer and the viability was measured over a period of 5 h (Fig. 2e). Minimum effect of BRK buffer on yeast viability was observed over a period of 5 h.

Concentration and viability results

Using the BRK buffer and the yeast staining AOPI solution, the concentration and viability of each YCM sample were measured using the imaging cytometry system, and the results were compared with those obtained using the hemocytometer counting method. Bright-field and fluorescence images of the seven yeast samples are shown in Fig. 3. Each fluorescence image is an overlay of the fluorescence signals from FL channel 1 (viable) and FL channel 2 (nonviable), presented using green and orange false colors, respectively. The counted yeast cells were circled by the software, which clearly distinguishes the viable cells, nonviable cells, and debris. The yeast cells were effectively stained with high fluorescence intensities, while the corn

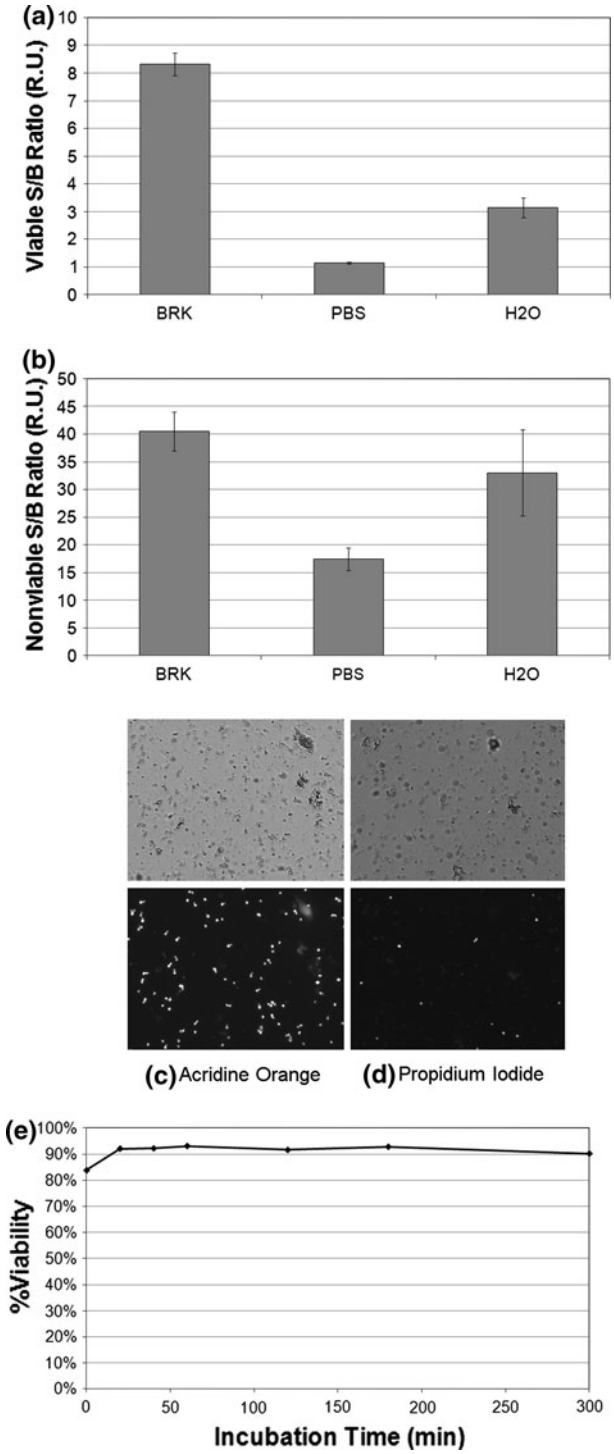

Fig. 2 Buffer selection for yeast enumeration in corn mash. The fluorescence signal-to-background (S/B) ratio is a representation of signal size of yeast over background fluorescence. Both **a** viable and **b** nonviable yeasts showed highest S/B ratio for the BRK buffer. The BRK buffer enhanced fluorescence intensity of yeasts and reduced nonspecific fluorescence of corn mash (**c**, **d**), which enabled automated enumeration. The fluorescence detection of yeast diluted in PBS and H_2O resulted in weak fluorescence intensity and high corn mash nonspecific signals, similar to results shown in Fig. 1. **e** Minimum effect on viability of yeasts during 5 h of incubation in the BRK buffer, confirming the stability of the buffer

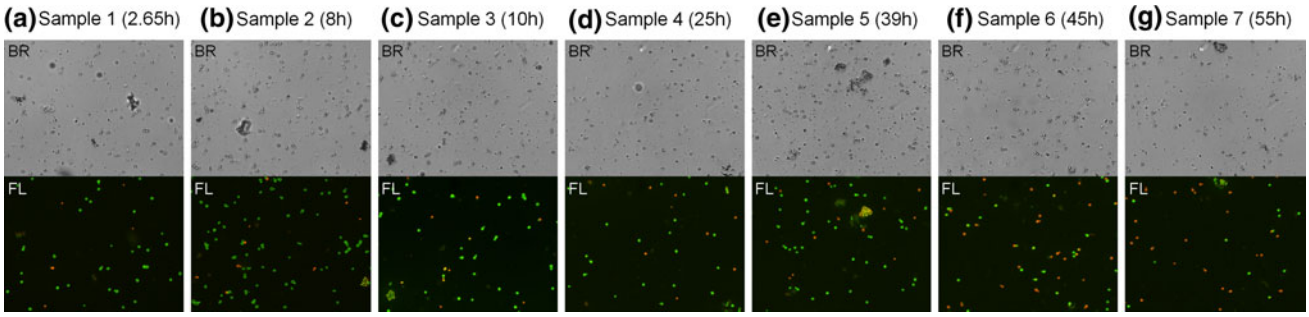

Fig. 3 Bright-field and fluorescence images of the seven YCM samples. YCM samples were collected directly from operating fermenters at seven time points in a standard fermentation process. Each sample was diluted in the BRK buffer and stained with the yeast staining solution. Concentration and viability were determined by analyzing sequentially captured bright-field and fluorescence images of stained samples on disposable counting slides using the Cellometer® instrument. Visually, the number of viable cells decreases, while nonviable cells increase in the later hours of the fermentation process, correlating with the calculated viability results. The nonspecific fluorescence signals of corn mash are minimized, which allowed the software algorithm to easily count only the viable and nonviable cells, disregarding the debris

Table 1 Concentration and viability results for the seven YCM samples

	Sample 1 (2.65 h)	Sample 2 (8 h)	Sample 3 (10 h)	Sample 4 (25 h)	Sample 5 (39 h)	Sample 6 (45 h)	Sample 7 (55 h)
Concentration (particles/ml)							
Cellometer®	1.16E + 08	1.35E + 08	1.31E + 08	1.21E + 08	1.19E + 08	1.09E + 08	8.33E + 07
Hemocytometer	1.10E + 08	1.20E + 08	1.23E + 08	1.25E + 08	1.21E + 08	1.06E + 08	8.28E + 07
Viability (%)							
Cellometer®	88.8	90.9	83.6	78.4	80.8	60.9	24.2
Hemocytometer	90.5	87.2	86.7	82.2	74.6	62.0	16.5

The results were consistent between the imaging cytometry method and hemocytometer

mash nonspecific signal was minimized. Since the YCM samples were diluted 1:20 by volume with the BRK buffer, the concentration results generated by the software were automatically multiplied by a factor of 20. The concentration and viability results for each YCM sample are shown in Table 1 and Fig. 4a, b for the imaging cytometry and hemocytometer methods. The seven YCM samples exhibited an average of approximately 1.16×10^8 and 1.12×10^8 particles/ml for the imaging cytometry method and manual counting, respectively, illustrating the consistency between the two methods and confirming that the imaging cytometry method can generate accurate results similar to the industry-standard method. The concentrations of the seven YCM samples appeared to decrease over the fermentation time course, which may indicate cell death due to unfavorable growth conditions. It was observed that both methods showed a dramatic decrease in viability after 55 h of fermentation, which marked the end of the normal fermentation cycle in the process. It is important to note that the 20 times buffer dilution with YCM samples reduced the viscosity of the corn mash solution, which made it easier to pipette the testing volume of 10 µl.

Although fluorescence detection of yeasts has been well studied in the past, previous publications reported mainly on enumeration of pure yeast samples. The current biofuel industry method still relies on manual counting using methylene blue, but it is difficult to automate this method. The ability to automatically measure yeast concentration and viability directly from fermenters is significant for the biofuel industry in the USA. This imaging cytometry method could be performed in biofuel plants to standardize the concentration and viability of yeasts for each fermentation process, which could improve consistency in multiple fermentation runs in different fermenters. Another advantage is the reduction of time spent on manual cell counting. Since the imaging cytometry counting is automated, trained technicians can easily set up and run multiple samples while obtaining results similar to manual counting. Furthermore, the imaging cytometry method usually counts thousands of cells, which can improve the accuracy of the results. One major concern is the applicability of this method. The experimental data presented in this work pertain only to one yeast and corn mash type from Lincolnway Energy, but different yeast strains and corn mash ingredients may also affect the fluorescence intensities. Therefore, different fermentation samples may require adjustment to dye concentration and buffer solution to optimize the counting

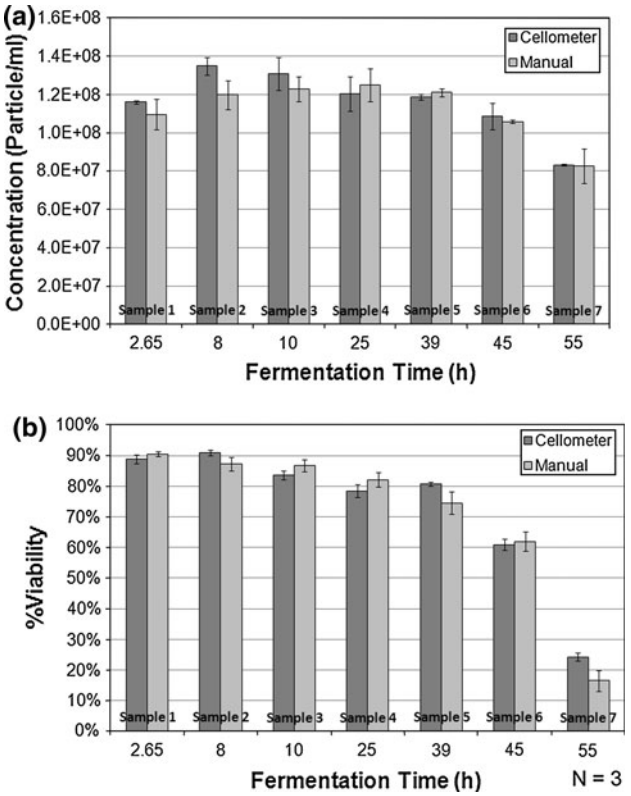

Fig. 4 Concentration and viability results as a function of fermentation process time. Results were obtained by both imaging cytometry method and manual hemocytometer. **a** Total yeast concentration, calculated by summing the viable and nonviable cell concentrations. **b** Yeast viability, calculated by dividing the nonviable cell concentration by the total concentration

method. Further research on the effectiveness of this method using various yeast strains and fermentation conditions should be performed.

We have demonstrated the capability to determine yeast concentration and viability in corn mash using a novel imaging cytometry method. Strong fluorescence signals are detected from viable and nonviable yeast cells, while nonspecific staining of corn mash is minimized, enabling effective automated cell counting. The development of a counting protocol employing the imaging cytometry instrumentation provides a useful tool to monitor yeast concentration and viability rapidly for quality control throughout the fermentation process. This method has the potential to improve yeast research. By studying the viability of various yeast strains under different fermentation conditions, one can identify higher ethanol-tolerant yeast strains that could optimize bioethanol production. In addition, viability can be studied to understand whether enzymatic activity of nonviable yeast can further increase bioethanol output. Furthermore, the method can potentially be applied to counting yeast in sugar cane and other complex media, which will have a tremendous impact on renewable energy development as the issues of fossil-fuel depletion and global warming become increasingly important.

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

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