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Scientific Note

Development of an Epi-Fluorescence Assay for Monitoring Yeast Viability and Pretreatment Hydrolysate Toxicity in the Presence of Lignocellulosic Solids

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Index Entries: Viability; fluorescence staining; *Saccharomyces cerevisiae*; hydrolysate toxicity.

INTRODUCTION

Biochemical conversion of cellulosic renewable resources to ethanol uses cellulolytic enzymes to hydrolyze the polymeric carbohydrates of the biomass to soluble sugars and subsequently ferment the sugars to ethanol using ethanologenic microorganisms. The cellulose reactivity is increased significantly if the biomass is pretreated by dilute acids (0.5–1% w/w sulfuric acid) at high temperatures (160–200°C) *(1)*. This process solubilizes most of the hemicellulose and part of the lignin in the biomass and, thus, improves the accessibility of the cellulosic matrix to cellulase enzymes. However, the solubilized sugars are unstable under the pretreatment conditions and degrade to furfural and 5-hydroxymethyl-2-furaldehyde (HMF) *(2)*. These sugar degradation products, along with the hemicellulose-derived acetate and phenolic lignin fragmentation products, inhibit cell metabolism *(3)* and might detrimentally affect fermentation performance. Therefore, monitoring the activity of the biocatalysts is fundamentally important in evaluating the performance of the conversion process and in estimating process yields *(4)*.

The activity of the cellulolytic enzymes in the liquid phase can be measured by standard assays *(5)*. The cell's biochemical activity is usually based on either dry cell weight (DCW) measurements, which measures total cell mass, or colony forming unit (CFU) measurements *(6)*, which measures the number of active cells. Moreover, the presence of lignocellulosic solids further complicates DCW determination, making it impractical to apply this method to a simultaneous saccharification and fermentation (SSF) process. Sonnleitner and coworkers *(7)* and Kennedy and coworkers *(8)* have recently reviewed several techniques that have been pro-

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posed and applied with varying degrees of success to alleviate the aforementioned problems with solids fermentations. More recently, additional reports have appeared that describe optimizing conventional methylene-blue-based methods *(9)*, and developing new techniques based on intracellular pH (proton pump activity) *(10)* and intracellular NADH (metabolic activity) *(11)* to determine yeast cell viability. Some have potential for on-line application *(12)*.

A viability assay can be of practical value in monitoring large-scale SSF process performance if:

1. The assay is selective and based on measure of a cell-specific property;
2. The measurement is not affected by the presence of solids;
3. The assay can be automated to some degree to allow rapid processing of a large number of samples; and
4. The technique has potential for on-line application for automated process monitoring.

A class of techniques, based on cell-specific fluorescence measurements, seem to meet most of the above criteria *(13,14)*. In this article, we explore the use of a fluorescence-based yeast viability assay as a tool for monitoring SSF performance. The assay is based on a commercial staining kit *(15)* that exhibits high specificity for metabolically active yeast cells. We discuss the development of the staining protocol, the effect of solids, and the application of the assay to develop a rapid method for assessing pretreatment hydrolysate toxicity.

MATERIALS AND METHODS

Organism and Growth Conditions

Saccharomyces cerevisiae D_5A *(16)* was used in all experiments. The culture was grown from frozen glycerol stocks in YEPD medium (2% w/v peptone, 1% w/v yeast extract, and 2% w/v dextrose). The medium was adjusted to pH 5.0 with sulfuric acid. The culture was incubated in baffled flasks at 37°C in a gyrotory shaker at 150 rpm. In all assays, the culture had reached stationary phase at a concentration of 10^8 cells/mL. For the assays that used nonviable cells, the yeast culture was kept at 37°C in the gyrotory shaker for 10 d after glucose was depleted.

Staining Protocol and Fluorescence Measurements

The Live/Dead *Fungo*Light Viability Kit containing a 10-m*M* stock solution of Fun-1 viability stain was purchased from Molecular Probes, Inc. (Eugene, OR). Fun-1 dye penetrates live and dead cells by passive diffusion. Once the dye is in the cells, only respiring yeast and fungi can chemically modify the Fun-1 dye from a diffuse green fluorescence in the cytoplasm to orange-red fluorescent structures. Dead cells stain a bright yellow-green with no red structures, and metabolically inactive cells remain a diffuse fluorescent green *(15)*. Only one dye is needed for the viability assay. There are no washing or destaining steps involved, so the stain is very easy to use. The two-color stain allows viable cells to be quantified using microscopy, an automated fluorometer, or an automated fluorescence plate reader.

The basic staining protocol *(15)* begins with harvesting the yeast culture (10^7–10^8 cells/mL), centrifuging the cells, and resuspending in 0.2 μ*M* filtered 10 m*M* sodium HEPES buffer with 2% w/v glucose (GH buffer). An aliquot of the washed cells is transferred to a new tube. Fun-1 dye, diluted in GH buffer, is added

to the tube and the mixture is incubated at 37°C for 30 min. After incubation, the number of viable cells possessing the orange-red structures and the number of bright or diffusely green cells can be counted using an epi-fluorescence microscope equipped with 485/530-nm excitation/emission long-pass filter set. The percentage of viable cells can be determined from the total number of cells counted. An automated fluorescence plate reader, equipped with 485/530- and 620-nm excitation and emission filter sets can also be used to automate the tedious counting step. Viability is determined by reading the red and green fluorescence from the sample, subtracting the background fluorescence contributions from the free dye and the solids, and calculating the ratio of the corrected red to green readings. This viability ratio should be correlated to the percent viable cells.

Optimizing the Staining Protocol

We further optimized the basic staining protocol to maximize the cell-specific red fluorescence and minimize the nonspecific background staining in the presence of lignocellulosic solids. The amounts of dye, cells, and solid substrate must be optimized because different solid substrates absorb different amounts of the dye. In determining the concentration of dye with yeast cells alone, 0.2 mL of cells was centrifuged for 2 min at 10,000*g* and resuspended in 1 mL of GH buffer. A volume of 0.1 mL of a 200-μ*M* Fun-1 solution was prepared from the 10-m*M* stock and diluted by twofold serial dilutions in GH buffer to yield concentrations in the range of 1.6 to 100 m*M*. An equal volume of the cell suspension was added to each tube, resulting in final dye concentrations of 50, 25, 12.5, 6.2, 3.1, 1.6, and 0.8 μ*M* and a cell concentration of 10^7 cells/mL. The tubes were incubated at 37°C for 30 min after which the stained cells were examined under a Nikon microscope equipped with an epi-fluorescence light source and a 430/515-nm excitation/emission long-pass filter set to assess the production of orange-red structures and determine the extent of nonspecific staining.

The staining protocol was further optimized in the presence of SSF solids. Based on previous experience with the methlyene blue stain, we used a waste-paper SSF slurry because it is more absorbent than poplar sawdust or corn stover. The yeasts were inoculated into a flask containing 5% w/v waste paper, cellulase enzyme, and nutrients, and incubated for 72 h before a sample was taken. We followed the same basic staining procedure stated above: 0.1 mL of the washed, resuspended slurry was added to an equal volume of diluted Fun-1 solutions to yield final dye concentrations of 3.1, 1.6, and 0.8 μ*M*. To compare to the standard staining procedure above, we increased the dilution of the SSF samples stained by reducing the volume of harvested SSF slurry to 0.04 mL. We also included SSF samples that were spiked with pure yeast culture. The samples were incubated at 37°C for 30 min and then examined under the epi-fluorescence microscope for production of orange-red structures and for nonspecific staining of the solids.

Determining Cell Viability in the Presence of Lignocellulosic Solids

To assess the potential for automating the yeast viability assay in the presence of lignocellulosic solids, we evaluated its sensitivity by comparing the viability ratio of viable and dead yeast samples when mixed with three types of lignocellulosic solids: partially hydrolyzed waste paper, sawdust, and corn stover, at a 5% w/v solids level. We used the basic staining procedure described above. Twenty microliters of live or dead yeast were incubated with an equal volume of the three lig-

nocellulosic solids in 3.1 μ*M* Fun-1. GH buffer with dye solution, and each substrate with the dye were included as negative controls. Quantitative fluorescence data were acquired with the Millipore Cytofluor 2350 (Millipore, Bedford, MA) microplate reader. A 485-nm excitation filter with a half-band width of 20 nm was used with three emission filters: a 530-nm with a half-band width of 25 nm (green), a 590-nm with a half-band width of 35 nm (red), and a 620-nm with a half-band width of 40 nm (red). Cytofluor 96-well titer plates (Millipore) were used in all experiments. After incubating the samples for 30 min at 37°C, the amounts of red and green fluorescence were determined for each sample and blanks. The red and green fluorescence from each blank was subtracted from the respective samples. The ratio of corrected red to green fluorescence was calculated to obtain the viability ratio.

Hydrolysate Toxicity Assay

To assess the toxicity of pretreatment hydrolysates, the viability ratio of yeast cells incubated in pretreatment hydrolysates at varying concentrations was used as a toxicity indicator. The pH of each hydrolysate was adjusted to 5.0, and 2% w/v glucose was added to each hydrolysate before testing. One milliliter of washed yeast culture was distributed to 0.1-mL aliquots in microfuge tubes and centrifuged (10,000*g* for 2 min). The supernatant was carefully removed, and the hydrolysates were added at 10, 20, 40, 60, 80, and 100% concentrations to the pelleted cells. A GH buffer blank, along with a blank for each hydrolysate at the various concentrations, was included. We added 0.031 mL of the 200-μ*M* Fun-1 solution to each sample, including the blanks. The final working volume for each tube was 0.2 mL. The samples were incubated for 30 and 60 min at 37°C. After incubation, the samples were transferred to a 96-well titer plate. The amounts of red and green fluorescence were read using the microplate reader with the same filter sets described above.

RESULTS AND DISCUSSION

Optimizing the Staining Protocol

Because different solids absorb different amounts of the dye, the concentration of dye used needs to be determined as a function of cell concentration and type and concentration of the solids present. A maximum production of orange-red structures produced by the viable cells and a low background (bright green) that would interfere with automated fluorescence measurements are desirable. In the first part of the optimization procedure, we fixed the cell concentration and varied the dye amounts. Yeast cells were stained with 0.8 to 50 μ*M* Fun-1 dye and examined under the epi-fluorescence microscope for orange-red structures and nonspecific staining. At 50, 25, and 12.5 μ*M*, no orange-red structures were observed because the stain concentration was too high, resulting in bright green nonspecific staining of cells. At 6.3-μ*M* concentration, there was reduced nonspecific staining, but the orange-red structures were not easily observed. The concentrations, 3.1 and 1.6 μ*M*, yielded the best production of orange-red structures with little nonspecific staining. At 0.8 μ*M*, the concentration was too low to see much staining. For 10^7 cells/mL, a 3.1-μ*M* concentration was chosen.

In the second part of the optimization process, we used samples from a waste-paper SSF to determine optimal dye, cell, and solid concentrations. The best combinations of dye and solids that produced distinguishable orange-red structures

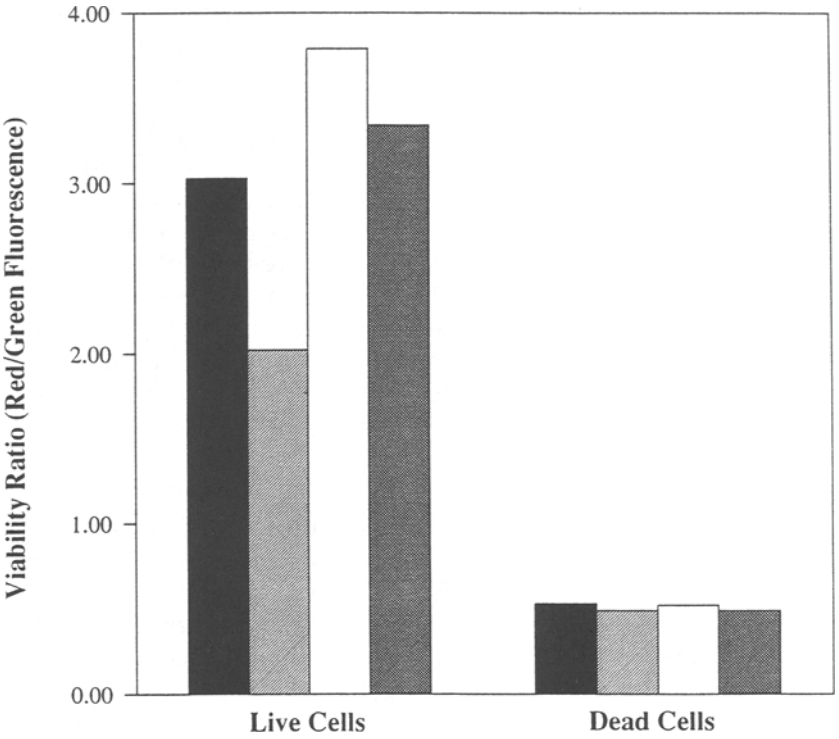

Fig. 1. Comparison of the viability ratio of *S. cerevisiae* D_5A in the presence of lignocellulosic solids. Live and dead yeast cultures were incubated with the Fun-1 viability dye for 30 min at 37°C without solids (■), with waste paper (▨)with sawdust (□), and with corn stover (▩). To determine the viability ratio, the red fluorescence was quantitated with a fluorescence microplate reader with 480/610-nm excitation/emission filters and the green fluorescence quantitated with 480/530-nm filter sets.

and a low background staining of the solids were 3.1 or 1.6 μ*M* with either 0.04 mL of paper SSF slurry or 0.02 mL of paper SSF with 0.02 mL of added yeast cells. Increasing the amount of cells to 0.2 mL and adding that to 0.2 mL of paper SSF resulted in fewer orange-red structures, because the dye was mostly absorbed by the increased solids. If this stain is used with various solids, determining the optimal concentration is very important, because different lignocellulosic solids will absorb the dye differently.

Cell Viability in the Presence of Lignocellulosic Solids

Counting the viable yeast with the epi-fluorescence microscope can be very tedious, and the results can be biased by the investigator's interpretations *(17)*. Using an automated fluorescence plate reader to quantify the viability ratio could eliminate the manual counting step, provided that the automated assay is sensitive enough to distinguish between the green background from the solids and the orange-red from viable cells. Figure 1 shows differences in the viability ratio between live and dead yeast cells alone and in the presence of partially saccharified waste paper, saccharified sawdust, and corn stover. The viability stain is sensitive enough to distinguish between live and dead cells in the presence of solids.

However, a considerable variability in the viability ratio was evident among different substrates. We attribute this variability to differences in the absorbing capacity of the different substrates. Although the background green fluorescence contribution from each substrate was accounted for using appropriate blanks, substrates with greater absorbing capacity leave less dye available for staining of the cells. As a result, the number or the degree of staining of viable cells is reduced and, consequently, a lower amount of red fluorescence is measured. This could explain the lower viability ratio that was observed in the presence of waste paper.

When working with the viability stain in the presence of solids substrates, the staining procedure needs to be optimized for each substrate. Experimenting with different dye concentrations with each solid substrate may be necessary to achieve optimum cell staining. Work will also continue on relating the viability ratio to the actual percentage of viability for use in monitoring fermentations of lignocellulosic solids.

Hydrolysate Toxicity Test

Currently we evaluate the toxicity of pretreatment hydrolysates by adding glucose and nutrients to the hydrolysate and inoculating with a yeast culture into various levels of hydrolysate to determine the hydrolysate levels at which the cells will grow. Often the toxicity evaluations take several days to complete. Based on the same idea, we used the viability dye with the fluorescence plate reader to determine the viability ratio of yeast cells incubated at varying concentrations of hydrolysate. Three pretreatment hydrolysates, with varying degrees of toxicity, were chosen for the toxicity test. From our previous tests, the batch pretreated paper hydrolysate was the least toxic, supporting growth up to 50% v/v concentration. The percolation pretreated paper hydrolysate and the batch pretreated wood hydrolysate were toxic at 20% v/v concentration. By plotting the viability ratio at the different hydrolysate concentrations, we can determine the concentration at which the hydrolysate begins to have toxic effects on the cells. The slope of the curve indicates the potency of the toxic effect. Figure 2 shows the viability ratio when yeast cells are incubated for 30 min in varying concentrations of each of the three hydrolysates. The viability ratio does not drop off as quickly with the batch paper hydrolysate as it does with the other two hydrolysates, indicating that this hydrolysate is less toxic to the yeast. This agrees well with our previous observations. Figure 3 shows the viability ratio when the cells are incubated for 60 min in the hydrolysates. The trends were the same for both incubation times, so any toxicity effects can be seen with just a 30–min incubation. If we could correlate the percent viability to the viability ratio and then determine the concentration of hydrolysate where 50% of the cells are viable, we would have a quick, quantitative way of evaluating different pretreatment processes. Also, by using the same organism and process conditions, this assay has direct correlation to the actual fermentation process.

CONCLUSIONS

Determining yeast viability is straightforward in liquid cultures, but not so when lignocellulosic solids are present. The Fun-1 viability dye is a fluorescent stain, which only viable yeasts chemically modify to produce a red color. Solid substrates have no effect on the dye changing color. Therefore, we used the dye to stain yeast cells with waste paper, sawdust, and corn stover. We found that optimiz-

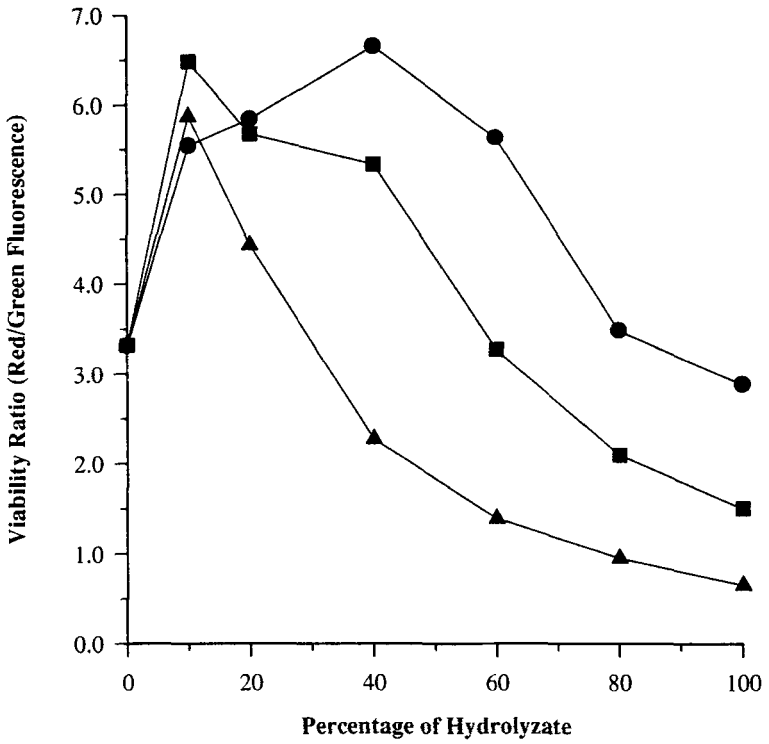

Fig. 2. Effect of three types of lignocellulosic pretreatment hydrolysates on the viability ratio when *S. cerevisiae* D_5A was incubated for 30 min in each hydrolysate. The three hydrolysates were generated from batch pretreated waste paper (●), percolation pretreated wastepaper (■), and batch pretreated wood (▲). Two percent (w/v) glucose was added to each hydrolysate, and the pH was adjusted to 5.0 with NaOH before incubation. To achieve the appropriate dilutions, the hydrolysates were diluted in Na-HEPES with 2% w/v glucose. Fluorescence was quantified with an automated fluorescence plate reader, as described in the text.

ing the amount of dye, cells, and solid substrate was an important first step, because the red fluorescence from the viable cells is affected by high background staining and by the solids absorbing the dye, thus making it difficult to detect viable cells. Once the staining procedure was optimized, we evaluated the use of an automated fluorescence reader to determine the viability ratio. By correcting for the background fluorescence from the solids, the viability ratio for the live cells with solids present was distinguishable from dead cells mixed with solids. Another application of the Fun-1 dye is its use in prehydrolysate toxicity assays. We have shown that by incubating the yeast in varying concentrations of hydrolysates with the Fun-1 dye, the viability ratio can be determined and plotted as a function of hydrolysate concentration for comparison to other hydrolysates. We used this assay to compare three hydrolysates with known toxicities to yeast cells. The viability results agreed well with our previous observations. This viability and toxicity assay is advantageous because the same organism and environmental conditions are used as in the actual fermentation process. Furthermore, the assay is rapid and not labor-intensive, so it would make a good screening tool for evaluating pretreatment conditions and for routine monitoring of pretreatment processes and equipment.

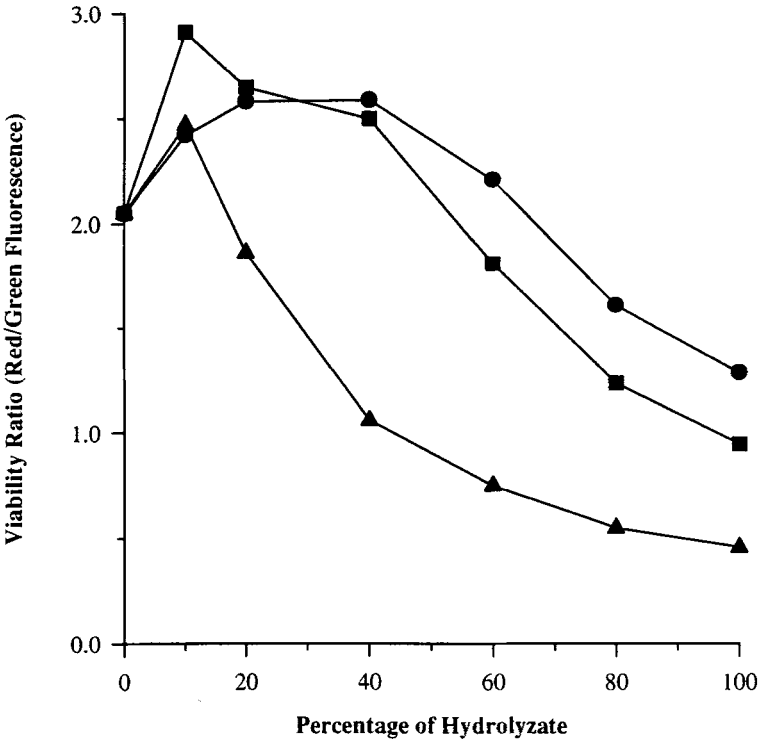

Fig. 3. Effect of three types of lignocellulosic pretreatment hydrolysates on the viability ratio when *S. cerevisiae* D_5A was incubated for 60 min in each hydrolysate. The three hydrolysates are the same as in Fig. 2. Measurements were carried out as described in Fig. 2.

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