Monitoring Growth of Beer Brewing Strains of *Saccharomyces Cerevisiae*

The Utility of Synergy™ H1 for Providing High Quality Kinetic Data for Yeast Growth Applications

Paul Held Ph.D., Principal Scientist, Applications Dept., BioTek Instruments, Inc., Winooski, VT

Key Words:

Yeast Growth

Kinetic Assays

Beer Fermentation

Strains of the yeast Saccharomyces cerevisiae serve critical roles in the production of many food staples such as beer, bread and wine. The fermentation of beer or wine yeast converts complex mixtures of sugars, amino acids, peptides, proteins, nucleic acids and numerous other compounds into alcohol, carbon dioxide and the desired flavors of the intended beverage. The ability to obtain the desired product reliably and repeatedly requires careful monitoring of not only the input materials, but the growth of the yeast strain during the process. Likewise the development of new strains often requires monitoring the growth of strains under various conditions. Here we describe the use of the Synergy™ H1 Hybrid Multi-Mode Microplate Reader to provide temperature control, solution agitation, and monitor cellular yeast growth using light scatter in 96-well microplates.

Introduction

Yeast are single celled eukaryotic fungi organisms that reproduce asexually by budding or division (Figure 1). While yeast can vary in size, they typically measure 3-8 µM in diameter. Saccharomyces cerevisiae is the most commonly used strain in scientific research, baking and fermentation and has become synonymous with the term yeast.

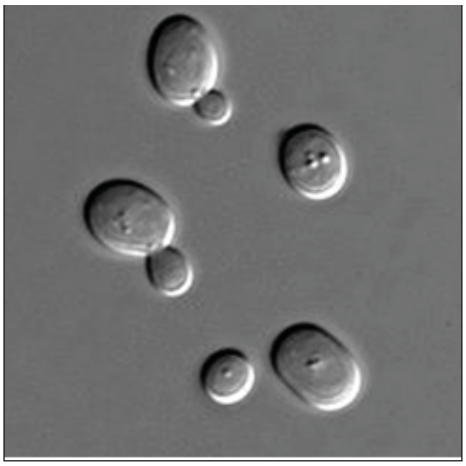

Figure 1. Saccharomyces cerevisiae.

Yeast has been used for thousands of years to ferment alcohol. The paintings discovered in the pharaoh's tombs depicting Egyptians using yeast for fermentation of alcoholic beverages and to leaven bread have documented the usage of yeast for at least 5000 years [1]. It wasn't until the 18th century that the biological sciences had advanced enough to identify yeast organisms and to deduce that sugars were fermented into ethanol with a carbon dioxide being emitted as a by-product. Since that time bakers, scientists and yeast manufactures have been working to identify strains of yeast that meet specific needs.

Yeast Growth Phases:

When cultured for the fermentation of beer, yeast cells in culture follow a very predictable pattern of growth that can easily be divided into four phases: (1) lag; (2) log; (3) deceleration; and (4) stationary. During the lag phase, no growth occurs as newly pitched yeast mature and acclimate to the environment. This is followed by the log phase, where cells are rapidly growing and dividing. Nutrients are in excess relative to cell number and waste is being sufficiently diluted as to be insignificant. The growth rate in this phase will follow first order kinetics. As cell numbers increase, cell growth begins to slow as a number of parameters (e. g. substrate and waste), each with saturation effects, become significant. Eventually the yeast cells reach the stationary phase, where no growth occurs due to high waste concentration or complete substrate consumption (Figure 2).

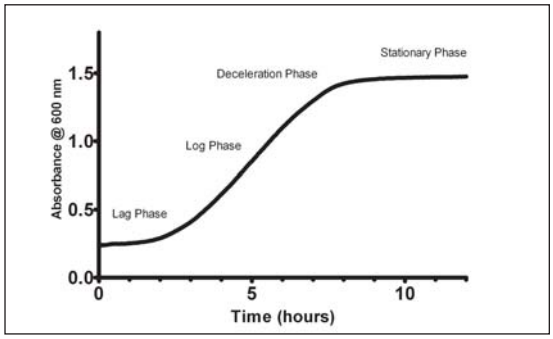

Figure 2. Typical Yeast Growth Curve. Saccharomyces cerevisiae grown in YPD media at 30°C for 12 hours with data measurements every 2 minutes.

BioTek Instruments, Inc.
P.O. Box 998, Highland Park,
Winooski, Vermont 05404-0998 USA
Tel: 888-451-5171
Outside the USA: 802-655-4740
E-mail: **customercare@biotek.com**
www.biotek.com

The clonal nature of budding yeast cell division allows for the screening of large numbers of organisms for mutants that exhibit desired characteristics. Individual mutants replicate and divide asexually from a single progenitor. When selecting yeast strains for desired growth characteristics, one method of doing so is to monitor growth under selective conditions. We have used the Synergy™ H1 Hybrid Multi-Mode Reader to maintain temperature, provide continuous orbital shaking, as well as perform absorbance measurements to monitor yeast cell growth under a variety of different conditions.

Materials and Methods

YPD media powder, sodium chloride, monobasic and dibasic phosphate, and ethanol were obtained from Sigma-Aldrich (St. Louis MO). YPD media was prepared as directed and sterilized by autoclaving. Sterile flat bottomed clear microplates, catalogue number 3598, were from Corning (Corning, NY). Optically clear TopSeal®-A plate sealers, catalogue number 6005185, were obtained from PerkinElmer (Boston, MA). Different strains of yeast strains were from Wyeast Laboratories (Odell, OR). All of the experiments followed the same general format. Overnight stock cultures (50 mL) were grown in 250 mL Erlenmeyer flasks at 30°C with orbital shaking at 125 RPM. Prior to growth experiments, 150 µL of each overnight stock culture was diluted to 7.5 mL with fresh 1X YPD media. The diluted cells were then plated as needed into Corning 3598 flat-bottomed clear plates. Measurements were made every 2 minutes with continuous orbital shaking mode employed. Shaking speed was set to slow and frequency set to 559 (1 mm amplitude). Temperature control was enabled and, unless stated otherwise, was at 30°C. Cell growth was assessed by light scatter measurements made at 600 nm [2]. The absorbance of each well was read kinetically using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). Reader was controlled and data collected using Gen5™ Data Analysis Software (BioTek Instruments, Winooski, VT).

Results

Effect of Cell Number

An overnight culture of strain 2000 Budvar lager yeast was diluted using YPD media as the diluent. Aliquots (200 µL) of each dilution were pipetted in replicates of 8 into a microplate and their absorbance at 600 nm was determined. The resultant absorbance measurements demonstrate an increase in absorbance with cell number (Figure 3). Because the cell suspension is being detected as a result of light scatter rather than true absorbance, the response does not obey Beer's law and is not necessarily linear. These data are best described by a second order polynomial.

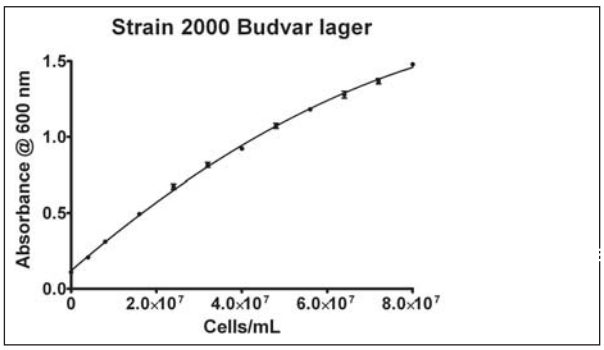

Figure 3. Absorbance of Strain 2000 Budvar lager yeast.

Growth Uniformity

Repeatable yeast cell growth across the entire plate is necessary for comparison studies. When a single strain 2000 Budvar lager yeast culture is plated in all the wells of a 96-well plate the growth patterns in all of the wells (Figure 4). As described previously, an aliquot of an overnight culture was diluted with YPD media and 200 µL was transferred to each well of a 96-well microplate. In these cultures, the average initial absorbance at 600 nm was 0.156 with CVs at 2.6%. After the completion of the experiment 20 hours later, the average absorbance across the plate was 1.516 with CVs at 1.5%. Individual column and row analysis showed similar results for both the initial and final absorbance results.

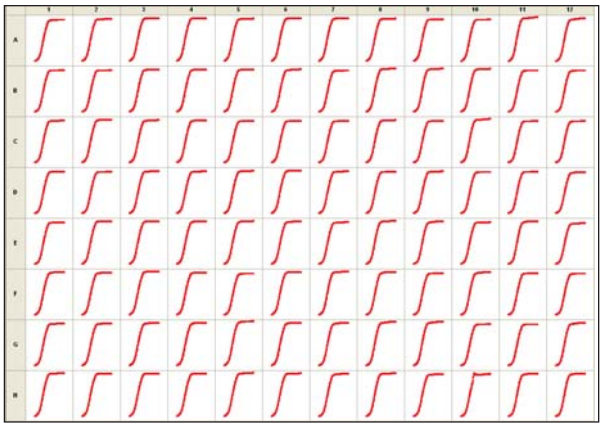

Figure 4. Kinetic Absorbance Patterns of equivalent strain 2000 Budvar yeast cultures.

When individual wells were examined in closer detail, very little differences between the wells can be discerned. This is true for not only the initial and final absorbance, but also the intervening data points as well (Figure 5). The average log-phase growth rate for all wells was 3.68 mOD/min., with a 2.48% CV. These data indicate that log-phase growth rates from different experimental conditions can be used as a means for comparison.

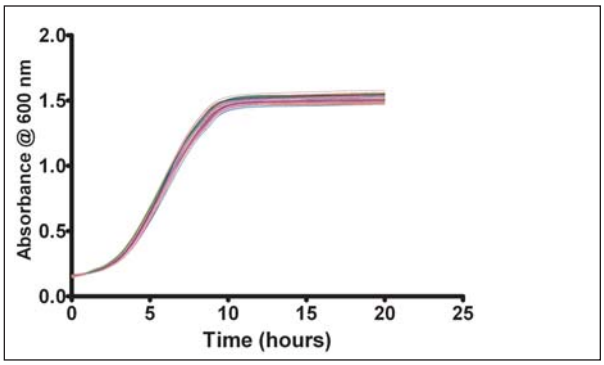

Figure 5. Comparison of yeast growth in select wells. The kinetic absorbance data was plotted for all 96 individual wells of strain 2000 Budvar lager yeast grown at 30°C.

Temperature Sensitivity

Growth temperature has a marked effect on the growth of yeast. Overnight cultures of four different strains of Saccharomyces cerevisiae were diluted and plated in replicates of 22 to six 96-well plates. Each plate had all four yeast strains and eight control wells that only contained YPD media. Plates were measured kinetically in separate readers at the temperature indicated. As demonstrated in Figure 6, Budvar lager yeast growth is virtually unchanged from Room temperature to 37°C. When cultured at 40°C, the lag time is slightly extended but, more importantly, the rate of growth during the log phase is decreased and the cell number at the stationary phase is lower than that observed with lower temperatures. Very little growth was observed when yeast cells are cultured at 45°C (Figure 6). Similar results were observed with other yeast strains (Data not shown).

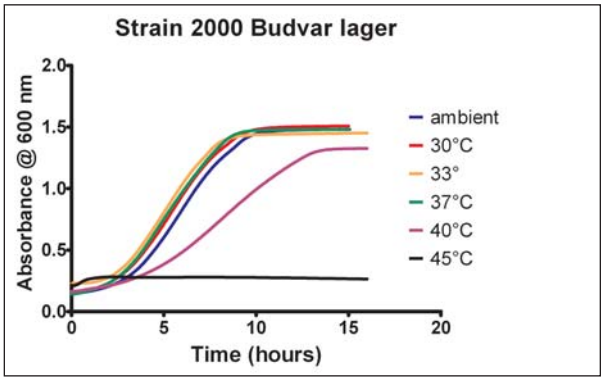

Figure 6. Effect of Temperature on the growth of strain 2000 Budvar lager yeast. The yeast strain 2000 Budvar was grown in parallel at the indicated temperatures.

By limiting the analysis to the log phase of growth comparisons of temperature resistance between different strains of yeast can be made. When log-phase growth rates, as measured by the change of absorbance per time, are plotted, significant differences in growth rate and temperature resistance are observed between different yeast strains (Figure 7).

As measured by change in absorbance, the two lager yeast strains had the fastest growth rate at temperature below 37°C. The two ale yeast strains (1098 and 1469) exhibited slower growth rates at permissible temperatures as well as less tolerance to increase in temperature as compared to the lager yeast.

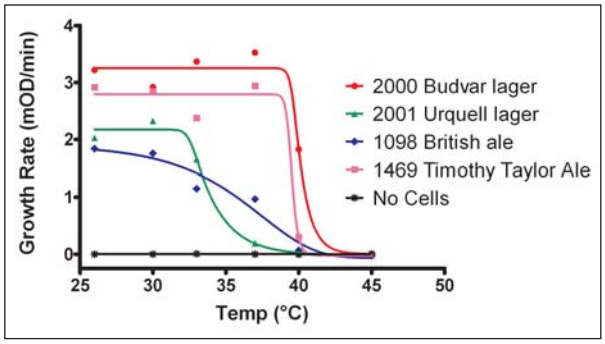

Figure 7. Effect of Temperature on Log phase Growth Rate.

Ethanol Tolerance

Yeast produces ethanol and carbon dioxide as byproducts of the fermentation of sugars. As the amount of waste becomes significant, its presence can become a detriment to cell growth. Overnight cultures of four different strains of Saccharomyces cerevisiae were diluted and 150 µL was plated in replicates of 22 into 96-well plate. An additional 50 µL of ethanol/YPD different media mixtures was added to the plate, such that each yeast strain was subjected to each ethanol mixture in replicates of two. When the influence of ethanol is examined, it becomes apparent that high levels of ethanol are inhibitory to yeast growth. Using the growth of strain 2001 Urquell lager as an example, yeast are quite tolerant to levels of 2.5% or less. Above these levels, a marked decrease in the growth of the yeast strain is observed (Figure 8).

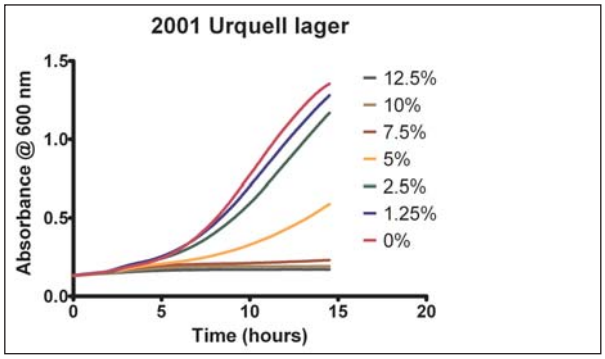

Figure 8. Effect of Ethanol on strain 2001 Urquell lager yeast growth.

Similar results are seen in the log-phase growth rates of the other yeast strains examined. Strain 2000 Budvar lager yeast is the most tolerant of the tested strains, showing no decrease in growth rate up to 5% ethanol. British ale was quite resistant to 2.5%, while the other two strains demonstrated a decreased rate with the presence of any ethanol (Figure 9).

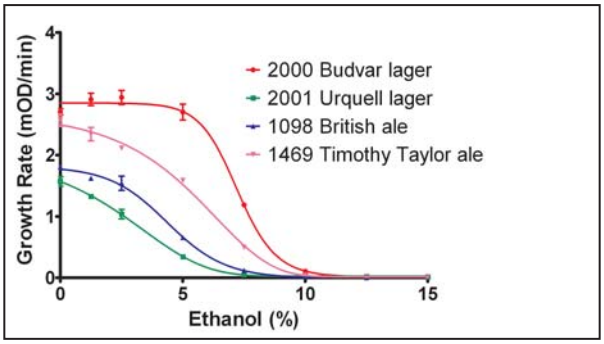

Figure 9. Automated Assay Performance data.

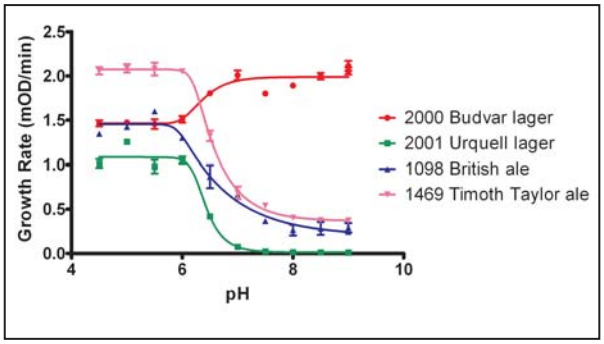

Figure 11. Effect of pH on log-phase Growth rates.

pH Sensitivity

In order to test the effect of pH on yeast cell growth, overnight cultures of four different strains of Saccharomyces cerevisiae were diluted and 150 µL was plated in replicates of 22 into a 96-well plate. An additional 50 µL of 50 mM phosphate at pH ranging from 4.5 to 9.5 were added to the plate, such that each yeast strain was subjected to different pH levels in replicates of two. Yeast has a propensity to grow much better in acidic conditions as compared to alkali. This is born out when the growth of strain 1469 Timothy Taylor ale yeast is examined. With pH levels from 4.5 to 6.0 the rate of growth is hardly distinguishable from the unbuffered control wells (Figure 10). At a pH of 6.5 the strain grows slower but reaches the same cell concentration at stationary phase, albeit it took 3-4 hours longer to achieve this level. At pH 7.0 or higher the strain shows a dramatic decrease in growth, both in terms of rate as well as final cell density.

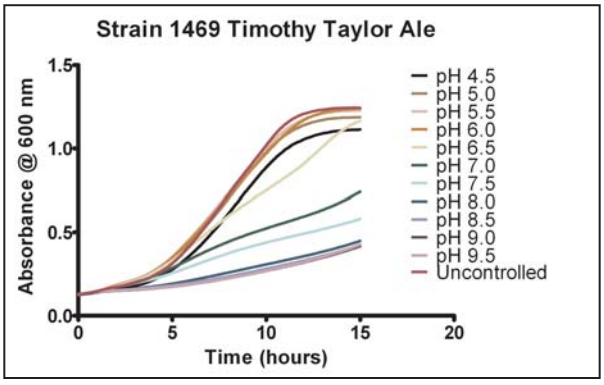

Figure 10. Effect of pH on the growth of strain 1469 Timothy Taylor ale yeast.

When other strains of yeast were examined, the results were quite mixed. Strains 2001 and 1098 showed similar patterns of growth as seen with strain 1469, while strain 2000 demonstrated an increase in growth rate at alkaline pH (Figure 11). Interestingly, the rates of all four strains were slightly lower than that recorded for the unregulated control wells at all pH levels tested (data not shown). This suggests that the presence of phosphate may have an over all inhibitory effect on yeast growth.

Salt Sensitivity

Ionic strength of the growth media is a concern when unprocessed water such as public water supplies or well water, as compared to deionized or distilled water, is used. While potable, these water types can contain large amounts of ions. While these "impurities" can actually add to the actual flavor of the water, they have the potential to add off flavors or inhibit yeast growth, depending on their makeup. Yeast cells show a great deal of tolerance in regards to growth in the presence of high sodium chloride concentrations. The effect of ionic strength on yeast cell growth was assessed by adding exogenous NaCl. As described previously, overnight cultures of four different strains of Saccharomyces cerevisiae were diluted and 150 µL was plated in replicates of 22 into 96-well plate. An additional 50 µL of NaCl dilutions was added, such that the final sodium chloride concentration ranged from 0 to 1.25M. Control wells received 50 µL of water. Each yeast strain was subjected to different sodium chloride concentrations in replicates of two.

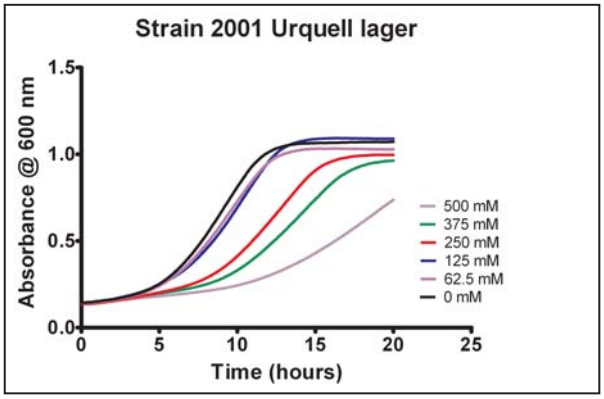

Figure 12. Effect of Sodium Chloride on the Growth of strain 2001 Urquell lager yeast.

Strain 2001 Urquell lager yeast grown in the presence of NaCl concentrations up to 125 mM show little visible change in growth patterns as compared to the untreated control wells. These concentrations, while hypo-osmotic, could be considered the equivalent of or an improvement to adding water only. Salt concentrations of 250 mM and 375 mM show a reduced growth rate, but still achieve similar cell density as the untreated controls by 20 hours. Significant growth of the yeast strain was seen in NaCl concentrations as high as 500 mM (Figure 12).

Log-phase growth rates of all four yeast strains for salt concentrations as high as 2.5M were calculated. Not surprisingly, all strains showed a decrease in log-phase growth rate with increasing NaCl levels (Figure 13). As was the case for many other experiments, strain 2000 Budvar showed the fastest growth rates at NaCl levels of 500 mM or lower. Interestingly, above 500 mM the ale yeast strain 1469 had the highest growth rate, albeit quite diminished from untreated controls of the strains tested.

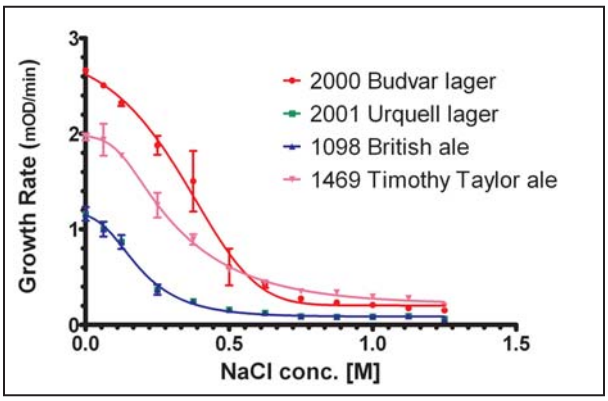

Figure 13. Effect of Sodium Chloride on Log-phase Growth Rate.

The strains of yeast used in these experiments have been selected over a number of years for specific attributes in regards to the fermentation of beer. Besides growth, characteristics such as clarity, sedimentation, flocculation, alcohol production (amount and type), flavor and aroma all play a role in selecting the strain. Historically, Northern Europe has produced lager beer that ferments at colder temperature, while the British Isles have produced ales. The 2000 Budvar lager strain demonstrated the greatest high temperature tolerance of the strains tested. While not tested, this suggests that it may also have an advantage at temperature levels lower than ambient. Likewise, the ability of the 1469 Timothy Taylor strain to have activity in the presence of high ionic strength solutions may very well be the result of the hard water often found in Great Britain.

Consistent results in these kinetic data are the product of several factors. Uniform temperature control across the entire plate is necessary for uniform growth of yeast in suspension, particularly at/or near the fall off point. The Synergy™ H1's four-zone heating system monitors the incubation temperature and provides heat from multiple points within the read chamber, providing uniform temperatures across the plate. An often overlooked point is evaporation and the need for plate sealers. Long kinetic assays such as these require a better means of evaporation prevention than can be provided with standard plate covers supplied with the microplate. The use of adhesive sealers is absolutely necessary to prevent fluid loss through evaporation.

The amount of fluid volume in the well is also an important parameter. An overfilled well often results in a small bubble forming between the plate seal and the fluid. This bubble will often cause large changes in the "absorbance" measurement as a result of light beam diffraction. With little fluid in the well a large air volume exists allowing a large amount of fluid to vaporize as the air gap becomes saturated with water vapor. Besides diminishing the fluid in which the yeast can grow, this also increases the likelihood of water condensing on the inner surface of the plate seal on the top of the well. These droplets will cause problems quite similar to the bubbles previously described. The incubation chamber of the Synergy H1 has heating elements on both the top and bottom of the plates which minimizes condensation formation. In our hands, 200 µL culture-volumes work well for yeast growth studies in 96-well flat-bottom plates.

The choice of shaking mode and speed is important in order to keep cells in suspension. The size and propensity of yeast cells to clump together necessitates the use of orbital shaking to maintain cells in suspension. With the strains we examined, a slow setting with a short-amplitude (1 mm) selected (559 cpm) provided adequate agitation to keep the yeast in suspension through all phases of growth. This is particularly important during the stationary phase, when yeast flocculates. Different yeast strains and/or different plate types would require different shaking settings than those described here.

These experiments used lengthy kinetic runs to analyze the capability of the yeast strains. An important point to note is that, despite markedly different growth rates, many of the different culture conditions resulted in the same final cell density. This is a finding that an endpoint determination at the end of a long incubation would have missed. The data analysis was simplified by the ability of Gen5™ to automatically determine average rate of change or MeanV on the log-phase kinetic data. The selection of the particular subset of data that constitutes the log-phase from the kinetic run can be made prior to the run and the calculations performed automatically on a subset of the kinetic data, allowing for the elimination of the lag and stationary phases.

These data demonstrate the utility of the Synergy™ H1 to monitor yeast cell growth kinetically. Yeast cultures require constant temperature along with adequate agitation for consistent repeatable results. Besides providing adequate mixing of the cellular nutrient suspension for cellular growth, maintenance of uniform suspension allows for accurate light scatter measurements.

The Synergy H1 has a number of different shaking features including linear, orbital and double or figure eight shaking modes. Each mode can also be adjusted for speed and amplitude, providing a number of different mixing options for different materials.

References

1. Jean-Luc Legras, Didier Merdinoglu, Jean-Marie Cornuet and Francis Karst. (2007.). "Bread, beer and wine: Saccharomyces cerevisiae diversity reflects human history". Molecular Ecology 16 (10): 2091–2102.

2. Stubbings, W.J., Bostock, J.M., Ingham, E., and Chopra, I., (2004) Assessment of a microplate method for determining the post-antibiotic effect in Staphylococcus aureus and Escherichia coli, J Antimicrobial Chemotherapy 54(1): 139-143.

Acknowledgements

We would like to thank Dr. Paul Hale for his kind donation of the yeast strains used in these experiments.