How environmental parameters affect low pressure fermentations assessed using image analysis techniques

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Introduction and Objectives

In this study the combinatory effect of extrinsic factors (including Free Amino Nitrogen (FAN), and temperature), on reduced pressure *Saccharomyces cerevisiae* fermentations were explored. Fermentation attributes including yeast growth, viability, and ethanol production were monitored using standard methods while yeast morphology was assessed using automated multivariate image analysis. Across all FAN and temperature levels, reduced pressure (vacuum pressure) fermentations resulted in a greater than or equal number of cells in suspension, higher average viability, and more ethanol production in comparison to atmospheric pressure fermentations, however the magnitude of the effect varied with extrinsic factors. The image analysis revealed that while yeast size was extremely variable across all fermentations, the ratio of vacuole to cell area consistently decreased over each fermentation and could be used to predict the point where yeast experienced a sharp decline in viability, subsequently ending the fermentation. This study showed that a combination of traditional measurements and novel automated analyses can be used by brewers to anticipate performance and endpoints of their fermentations, and that reduced pressure can have significant effects upon the rate and final ethanol concertation of variable industrial fermentations.

The Specific objective of this research was to assess the effect of reduced pressure on fermentations with variable FAN and temperature. Two different levels of each factor (high and low FAN concentration, temperature, and pressure) were selected based on the *Saccharomyces cerevisiae* strain used. Multivariant automated image analysis was used in an attempt to correlate physical changes with yeast health during fermentations under each set of factors. This study was performed to increase the understanding of yeast performance and morphology under various environmental conditions, allowing the brewing and distilling industry to apply these techniques to increase the fermentation process efficiency.

Methods

This research was designed to have similar conditions to Very-High-Gravity distiller's fermentation (Camargos et al, 2021) using molasses and sucrose to assess the effect of extrinsic factors on yeast performance. The experiments were designed by altering three extrinsic factors (FAN concentration, temperature, and pressure) with two levels for each (completed in duplicate). The fermentations were conducted in identical vacuum rated fermenters (Figure 1). The low FAN level obtained from the molasses used was 17 mg/L. Since the FAN requirement for fermentations with a high sugar concentration (> 18 % (w/w)) is reported to be 280 mg/L (Lei, Zhao, Yu, and Zhao 2012), yeast extract was added to the media to obtain 300 mg/L as the high FAN level. The effects of temperature are strain-dependent due to a complex heat-resistant response (Liu et al., 2019) that creates a "temperature range" where fermentation is possible. In this study, the lower and higher fermentation temperatures of the supplier recommended range (30 °C and 35 °C) were used for testing (referred to hereafter as low and high temperature). The low-pressure level selected was 24.1 kPa (vacuum), which was maintained throughout the fermentations by circulating water through a water aspirator connected to a centrifugal pump (Figure 2). The high pressure level was 101.3 kPa (atmospheric), as typically used in the brewing and distilling industry. All eight configurations of the experiments are presented in Table 1.

The combination of low temperature with high FAN (LTHF) was hypothesised to promote yeast growth with a high viability, resulting in faster fermentation with a high ethanol production. On the contrary, the combination of high temperature with low FAN was expected to hinder the yeast viability, resulting in a slow fermentation with a low ethanol production. Both conditions were expected to improve under vacuum pressure.

Yeast CSA and vacuolar ratio were assessed using the method described by Guadalupe-Daqui et al (2021). A sample with 0.01%(w/v) methylene blue solution was made to differentiate viable from dead cells. A total of 1 µL of sample was smeared onto a microscope slide using a Fisherbrand disposable inoculating loop (Pittsburgh, PA). Between 8 to 13 microscope images for every sample were captured using a Nikon Eclipse Ci-L microscope at 1000× magnification, combining a 100× oil immersion objective and a 10× eyepiece. ImageJ v1.8.0 software (Madison, Wisconsin) was used to measure various cell properties including the average yeast cell CSA, total yeast CSA within an image, and the vacuolar CSA from between 200-600 yeast cells per sampling time. Figure 3 shows a graphic representation of the morphological measurements taken in this study.

Table 1: Setup of extrinsic factors under each fermentation configuration

Configuration	Extrinsic factors		
	Temperature (°C)	FAN (mg/L)	CO_2 Pressure (kPa)
Low temperature High FAN (LTHF)	30	300	24.1
High temperature High FAN (HTHF)	30	300	101.3
Low temperature Low FAN (LTLF)	35	300	24.1
High temperature Low FAN (HTLF)	30	17	24.1
	35	17	101.3

Figure 1: Vacuum fermentation vessels and sample collection chamber

Results and Discussion

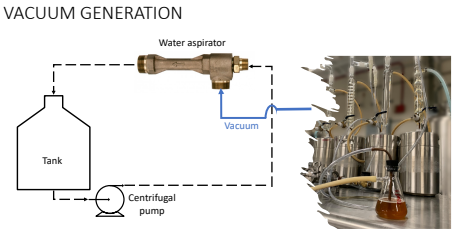

Figure 2: Graphic representation of apparatus used to generate vacuum during fermentation

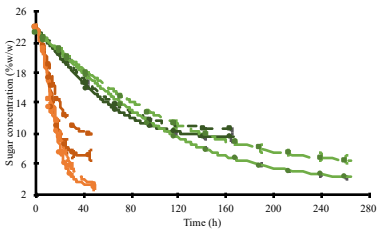

Figure 4: Sugar concentration throughout each fermentation configuration: LTHF (orange); HTHF (dark orange); LTLF (green); HTLF (dark green). Data shown represents sugar concentration under vacuum (circles) and atmospheric conditions (squares); sugar concentration model under vacuum (continuous lines) and atmospheric pressure (dotted lines); high FAN (filled), Low FAN (empty); high temperature (darker shade) and low temperature (lighter shade).

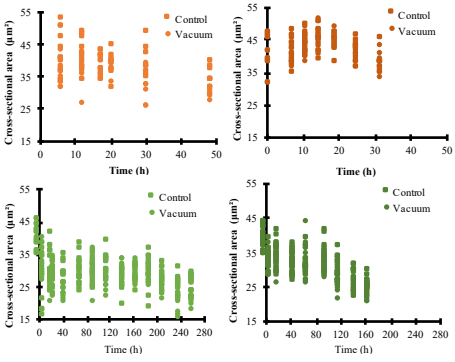

Figure 5: Average overall yeast cell CSA plotted against time: (a) LTHF (orange); (b) HTHF (dark orange); (c) LTLF (green); (d) HTLF (dark green). Fermentations were performed in duplicate. Data shown represents yeast cell CSA under vacuum (circles) and atmospheric pressure (squares); high FAN (filled) and low FAN (empty); high temperature (darker shade) and low temperature (lighter shade); viability (crosses).

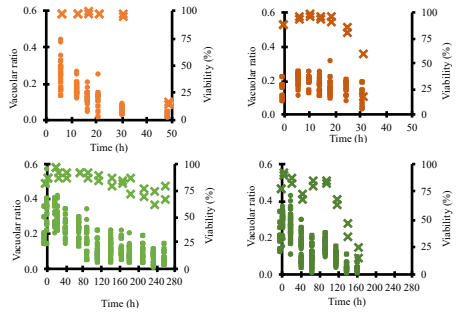

Figure 6: Vacuolar ratio (total vacuolar CSA to total yeast cell CSA) plotted over time: (a) LTHF (orange); (b) HTHF (dark orange); (c) LTLF (green); (d) HTLF (dark green). Fermentations were performed in duplicate. Data shown represents vacuolar ratio under vacuum (circles) and atmospheric conditions (squares); high FAN (filled) and low FAN (empty); high temperature (darker shade) and low temperature (lighter shade); yeast viability (crosses).

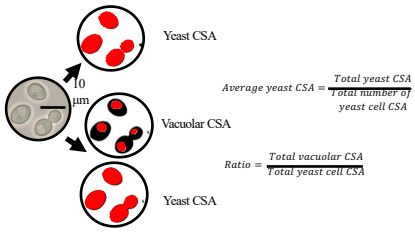

Figure 3: Graphic representation and calculation of the morphological properties: average yeast cell cross sectional area (CSA) and vacuolar ratio. The left circle image is a microscope image taken during fermentation (1000×). Right circle images are after image processing for the automated analysis with the red shading representing the variable collected.

Discussion

In this research, the three extrinsic factors of initial FAN concentration, pressure, and temperature were selected to assess their effect on fermentation attributes and yeast morphology. The parameter that had the most effect on ethanol concentration was initial FAN concentration (Figure 4), while the rate was most affected by temperature (as was expected from prevailing literature). When the pressure was reduced, the final ethanol concentration increased as did the fermentation rate for all but the low temp/high FAN condition. This finding was supported by literature that found vacuum condition improved fermentations when yeast stress was impacted by external conditions.

The yeast CSA was found to be higher when fermented with high initial FAN, high temperature, and atmospheric pressure (Figure 5). However, these results did not appear to indicate yeast CSA as a particularly reliable stress indicator by itself. An additional finding of this study was that as the fermentation progressed, the ratio of vacuole to cell size showed correlation with viability (Figure 6). The slope of the decrease was dependent upon external conditions, and as the fermentation was less optimal (high temp/low FAN), the slope increased.

Conclusion

These results can be used to optimize fermentations for particular aspects, including yeast biomass, rate, or final ethanol concentrations. They also suggest potential uses of multivariant automated image analysis as a tool for brewers, allowing quick identification of stress in yeast populations.

From these results we can conclude that brewers who desire a faster fermentation while not compromising total sugar consumption should consider fermenting under low temperature and high FAN concentration at atmospheric conditions (as vacuum pressure provided no benefits). However, brewers who would benefit from a slower fermentation (Gibson et al, 2017) can opt for a low temperature and low initial FAN concentration fermentation under vacuum to promote total sugar consumption.

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Citations

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