Effect of Cell Density and Growth Phase on Malolactic Fermentation

by *Oenococcus oeni*

Honor's Thesis
Presented to the College of Agriculture and Life Sciences
Department of Viticulture and Enology
Cornell University
in Partial Fulfillment of the Requirements for the Honors Research Program

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May 2011

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Abstract:

Oenococcus oeni is a species of lactic acid bacteria that is used in winemaking to perform malolactic fermentation, a conversion of malic acid to lactic acid. Not much is known about the impact of cell concentration and stage of growth on the ability of *O. oeni* to metabolize malic acid, but this information would be useful to winemakers who experience stuck malolactic fermentation. Corresponding optical absorbance at 650 nm and concentration data, based on viable cell plating, were collected for Alpha and MCW strains of *O. oeni* over time. This data was used to estimate viable cell concentrations and timing of growth phases for each strain using optical density measurements. Each stain was grown up to late stationary phase, washed and resuspended in buffer. Cells were then diluted to concentrations of 10^7 cells/mL, 10^6 cells/mL, 10^5 cells/mL, 10^4 cells/mL, 10^3 cells/mL, and 10^2 cells/mL and placed in a buffer that did not support growth. Malic acid was added, and samples were taken every half hour for four hours and analyzed for malic acid concentration by enzyme assay. This was repeated with cells that were harvested at the early exponential, mid exponential, late exponential/early stationary, and late stationary phases of growth at cell concentrations of 10^7 and 10^6 cells/mL. Results indicated that both strains performed malolactic fermentation from mid exponential to late stationary phase and that there was malolactic fermentation activity at both low and high concentrations (10^2 and 10^7 cells/mL). No conversion of malic acid occurred in cells harvested at the early stationary phase of growth.

Keywords: *Oenococcus oeni*, malolactic fermentation, quorum sensing, cell concentration, growth phase

Introduction:

Oenococcus oeni (O. oeni) is a species of lactic acid bacteria that is used in winemaking to perform malolactic fermentation, which converts naturally occurring malic acid to lactic acid. This takes place through the decarboxylation of L-malate to L-lactate, which benefits the cells energetically because the uniport of monoanionic L-malate assists in the generation of a proton motive gradient across the cell membrane that is critical to ATP production (Bauer and Dicks 2004). The result of malolactic fermentation in wine is generally a decrease in titratable acidity, an increase in pH, the conversion of harsh-tasting malic acid to more palatable lactic acid, and the contribution of aromatic compounds, such as diacetyl, that are also metabolic products of *O. oeni* (Van Vuuren and Dicks 1993). Because malolactic fermentation can decrease the perceived and actual acidity of a wine, it is very attractive to winemakers in cool climates, or winemakers in warmer climates that experience cooler years, where grapes do not have the opportunity to fully ripen and degrade the high levels of malic acid that naturally remain in immature grapes. In addition, malolactic fermentation can occur spontaneously in the bottle,

producing carbonation, changing the flavor of a finished wine, and forming an unsightly haze. Under these conditions, or under any circumstance where a decrease in wine acidity is undesirable, malolactic fermentation is considered a spoilage issue. This is typically only a problem in warmer regions where grapes have the potential to ripen so much that high pH wines, inviting to indigenous microflora, are produced. Performing an inoculated malolactic fermentation pre-bottling can prevent spontaneous malolactic fermentation by decreasing the nutrient levels in a wine and consuming available substrate (Pramateftaki et al 2006).

Inoculation cell concentrations for malolactic fermentation usually range between 10^6-10^8 cells/mL, because it is a commonly held belief in the wine industry that high cell concentrations increase the probability of rapid and complete malolactic fermentation (Maicas et al. 1999). Little is known about the ability of *O. oeni* to convert malic acid to lactic acid at lower concentrations, because a potentially slow or incomplete malolactic fermentation is impractical by industry standards. In addition, the current scientific literature does not address the rate or quantity of malic acid conversion preformed by *O. oeni* at different concentrations. It is known that malolactic bacteria are present in low concentrations on grapes, approximately 100 cells/g, but then they increase to 10^4 cells/mL after crush and 10^7 cells/mL by the end of alcoholic fermentation when malolactic fermentation typically occurs (Van Vuuren and Dicks 1993). It is possible that malolactic fermentation starts out at low cell concentrations, but the initial effects are not realized because it requires a sizable amount of malic acid conversion for a significant change in wine titratable acidity or pH to be observed. It is also possible that *O. oeni* can't ferment at low concentrations because the bacterium utilizes quorum sensing. Quorum sensing is a process used by delocalized bacteria to coordinate a response to changes in the environment. The release of a chemical signal triggers a change in gene expression, or metabolism, of cells in the bacterial population if the quorum threshold concentration is reached (Miller and Bassler 2001). Although quorum sensing has never been shown to occur in *O. oeni*, it is known that it occurs in other types of lactic acid bacteria such as *Lactobacillus plantarum* (Di Cagno et al. 2010). In addition, little is known about the ability of *O. oeni* to ferment at different stages of population growth. It is possible that some growth stages are conducive to malolactic fermentation, while others are not.

The first aim of this experiment is to determine whether resting *O. oeni* are capable of performing malolactic fermentation at low cell concentrations. The second goal of this study is to determine whether resting *O. oeni* are capable of malolactic fermentation at every stage of growth. Knowing whether there are growth stages or cell concentrations that prevent malolactic fermentation could explain why some malolactic fermentation are unsuccessful, and will provide winemakers with useful information for executing successful malolactic fermentation in the future.

Materials and Methods:

Stage I: Growth Curve Estimation

Alpha and MCW strains of *O. oeni* (Lallemand, Canada) were isolated as pure cultures and then grown in 10 mL test tubes containing malolactic broth medium (Cox and Henick-Kling 1995) at pH 3.5, a typical wine pH, in a 25° C incubator. Absorbance at 650 nm was measured until the cells reached stationary phase. Measurements were taken every day for days 1 and 2, twice per day for days 3 through 5 and once per day until a lack of further increase in absorbance indicated that the population was no longer growing exponentially. After absorbance was measured, 0.5 mL of each sample was collected immediately for viable cell estimations using serial dilution and spot plating on malolactic broth plates. All measurements were performed aseptically and in triplicate for each strain. A new tube was sampled each time to prevent contamination from affecting results. The average of the three absorbance readings and cell counts were calculated, and growth curves for absorbance vs. time, concentration vs. time, and concentration vs. absorbance were constructed to determine the suitability of using optical density as an estimation of viable cells/mL for later experiments. The equation obtained from the exponential growth portion of the growth curve for each strain was used to determine growth rate, and the growth curves were used to estimate at what point in time the bacteria would be in each stage of growth for subsequent experiments.

Stage II: Malolactic fermentation in resting cells at different concentrations

Alpha and MCW strains of *O. oeni* were grown up to late stationary phase in 0.5 L of malolactic broth at pH 3.5 in a 25° C incubator. The cell cultures were aseptically transferred into sterile centrifuge tubes and centrifuged for 10 min at 25° C at 13,000 rpm. The supernatant was decanted, and the cells were resuspended in sterile tartrate-phosphate buffer, containing 3 g/L D-tartrate (Sigma, St. Louis, MO), 60 mg $MgSO_4 \cdot 7 H_2O$ (Malinckrodt, Phillipsburg, NJ), 34 mg $MnSO_4 \cdot 4 H_20$ (Fisher, Fair Lawn, NJ) and 10.9 g KH_2PO_4 (Fisher, Fair Lawn, NJ), adjusted to pH 3.5. The suspension was then centrifuged again at the same settings. The supernatant was decanted, and the cells were resuspended in fresh tartrate phosphate buffer. This rinsing step was repeated twice. The cells were then resuspended in tartrate phosphate buffer a third time in a separate, sterile, flask to dilute until the concentration was low enough that it could be read on the spectrophotometer. Cell concentration was estimated from optical density based on the equation from the corresponding growth curve estimated in Stage I. Serial dilutions in tartrate phosphate buffer were performed to concentrations of 10^7 cells/mL, 10^6 cells/mL, 10^5 cells/mL, 10^4 cells/mL, 10^3 cells/mL, and 10^2 cells/mL. After that, 19 mL of each dilution was transferred to a sterile 50 mL flask, and 1 mL of malic acid (Sigma, St. Louis, MO) solution (80 g/L) was added to each suspension, for a final concentration of 4 g/L malic acid. The concentration of the 10^7 dilution was initially estimated using optical density and confirmed by plating for viable cell count on malolactic broth plates. The flasks were then incubated on an orbital shaker plate at 25°C at low speed to keep cells in suspension. A 0.5 mL sample was taken from each flask every 30 minutes over the course of 4 hours and immediately combined with 0.05 mL of 1.2 N perchloric acid (Malinckrodt, Phillipsburg, NJ) in a sterile microfuge tube to prevent further malolactic fermentation activity. The samples were then frozen until malic acid enzyme assays

could be performed. Malic acid enzyme assays were performed with a microplate reader using a Megazyme (Wicklow, Ireland) L-malic Liquid Ready Reagents kit. This experiment was performed in triplicate for each strain. Results were analyzed with JMP 9 statistical software and a value of $p<0.05$ was used to determine the statistical significance.

Stage III: Malolactic fermentation in resting cells harvested at different growth stages

Alpha and MCW strains of *O. oeni* were grown up in 0.5 L of malolactic broth at pH 3.5 in a 25° C incubator to the early exponential, mid exponential, late exponential/early stationary, and late stationary phases, based on optical density measurements and the growth curves constructed in Stage I. The cell cultures were aseptically transferred into sterile centrifuge tubes and centrifuged for 10 min at 25° C at 13,000 rpm. The supernatant was decanted, and the cells were resuspended in sterile tartrate phosphate buffer at pH 3.5. The suspension was centrifuged again at the same settings. The supernatant was decanted, and the cells were resuspended in fresh tartrate phosphate buffer. This rinsing step was repeated twice. The cells were resuspended in tartrate phosphate buffer a third time in a separate, sterile, flask and diluted until the concentration was low enough that it could be read on the spectrophotometer. Cell concentration was estimated from optical density based on the equation from the corresponding growth curve determined in Stage I. Serial dilutions in tartrate phosphate buffer were then preformed to concentrations of 10^7 cells/mL and 10^6 cells/mL. After that, 19 mL of each dilution was transferred to a sterile 50 mL flask, and 1 mL of malic acid solution (80 g/L) was added to each suspension, for a final concentration of 4 g/L malic acid. The concentration of the 10^7 dilution was initially estimated based on optical density and confirmed by plating for viable cell count. The flasks were then incubated on an orbital shaker plate at 25°C at low speed to keep cells in suspension. A 0.5 mL sample was taken from each flask every 30 minutes over the course of 3.5 hours and immediately combined with 0.05 mL of 1.2 N perchloric acid in a sterile microfuge tube, to halt further malolactic fermentation activity. The samples were then frozen until malic acid enzyme assays could be performed. Malic acid enzyme assays were performed with a microplate reader using a Megazyme L-malate Liquid Ready Reagents kit. This experiment was performed in triplicate for each strain. Results were analyzed with JMP 9 statistical software, and a value of $p<0.05$ was used to determine the statistical significance.

Results:

Stage I

The results for Stage I, an estimation of the growth curve of Alpha and MCW *O. oeni*, confirmed that optical density, based on absorbance at 650 nm, was a reliable estimate for viable cell counts (Fig. 1 and 2). This is supported by the 2005 Zhang and Lovitt study, which used a similar method to estimate population growth. In addition, the absorbance vs. time and concentration vs. time data for both strains indicated that it requires approximately four days for the bacteria to enter stationary phase (data not shown).

Fig. 1. Growth of strain Alpha: Comparison of viable cells/mL and absorbance measurements at 650 nm for Alpha *O. oeni* in malolactic broth at pH3.5 at 25° C.

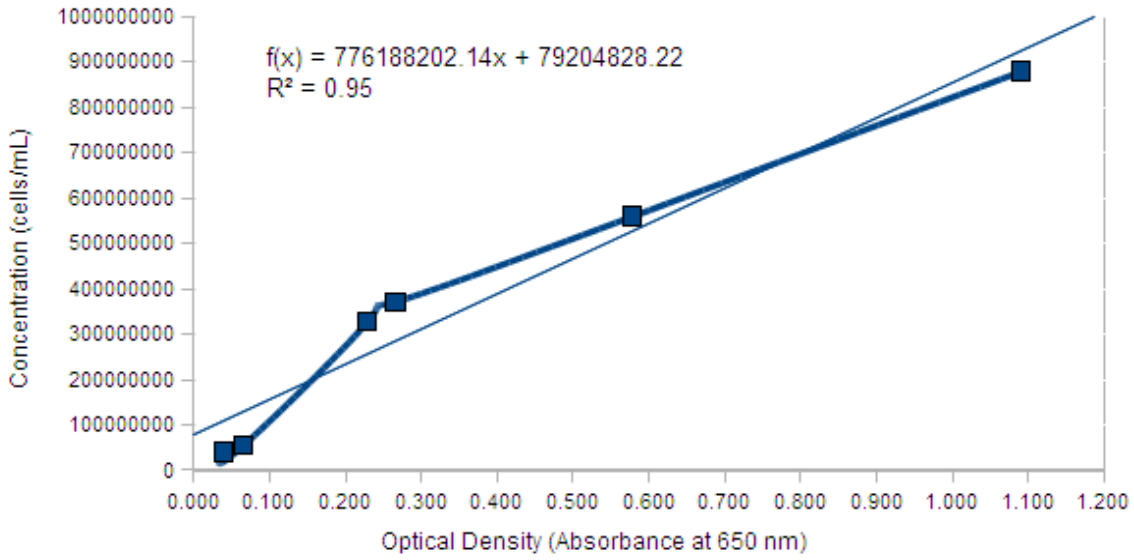
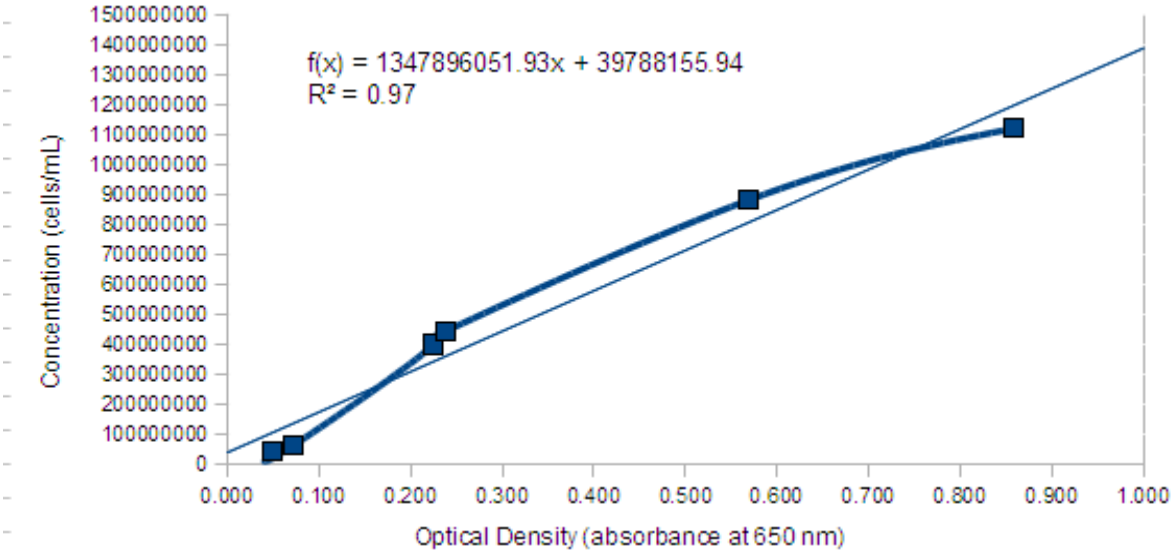


Fig. 2. Growth of strain MCW: Comparison of viable cells/mL and absorbance measurements at 650 nm for MCW *O. oeni* in malolactic broth at pH3.5 at 25° C.



Stage II

The results for Stage II, a study of resting Alpha and MCW *O. oeni* malolactic fermentation activity at different cell concentrations, showed a significant decrease in malic acid over time ($p<0.0001$) for all of the treatments. Individually, only Alpha at 10^3 and 10^7 cells/mL (Fig. 3 and 4), and MCW at 10^2 and 10^7 cells/mL (Fig. 5 and 6) showed a significant decrease in malic acid over time ($p=0.003$, $p=0.027$, $p=0.0210$, and $p=0.002$, respectively). The other treatments (Alpha 10^2, 10^4, 10^5, and 10^6 cells/mL and MCW 10^3, 10^4, 10^5, and 10^6 cells/mL), when examined individually, all showed a non-significant decrease in malic acid over time (Fig. 7). Rate of malolactic fermentation varied with concentration for both strains, but a consistent pattern of increase in rate of malolactic fermentation with increase in concentration was not observed for either strain. Unexplained fluctuations in the data were observed between the first and last measurements within treatments. These fluctuations are presumed to be the result of pipetting error during sample collection.

Fig. 3. Malic acid vs. time for Alpha 10^3 cells/mL: Malolactic fermentation activity ($p=0.003$) of resting Alpha *O. oeni* at 10^3 cells/ mL in tartrate phosphate buffer at pH 3.5 at 25° C over the course of 4 hours. The rate of fermentation can be described by the equation:

Malic Acid (g/L) = 2.1117262 - 0.0684365*Time (hr)

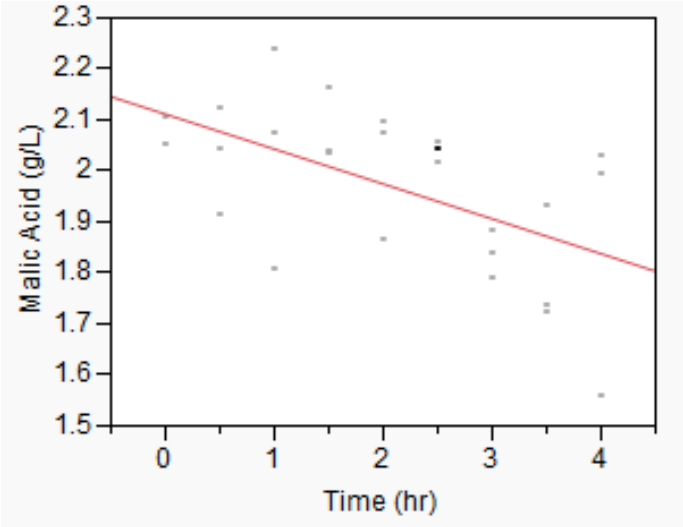


Fig. 4. Malic acid vs. time for Alpha 10^7 cells/mL: Malolactic fermentation activity (p=0.0027) of resting Alpha *O. oeni* at 10^7 cells/ mL in tartrate phosphate buffer at pH 3.5 at 25° C over the course of 4 hours. The rate of fermentation can be described by the equation:

Malic Acid (g/L) = 2.106771 - 0.0946972*Time (hr)

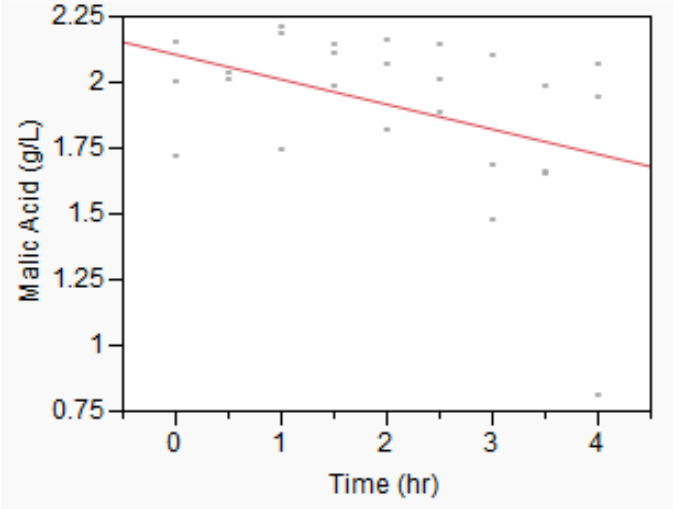


Fig. 5. Malic acid vs. time for MCW 10^2 cells/mL: Malolactic fermentation activity (p=0.0210) of resting MCW *O. oeni* at 10^2 cells/ mL in tartrate phosphate buffer at pH 3.5 at 25° C over the course of 4 hours. The rate of fermentation can be described by the equation:

Malic Acid (g/L) = 2.0732638 - 0.0620158*Time (hr)

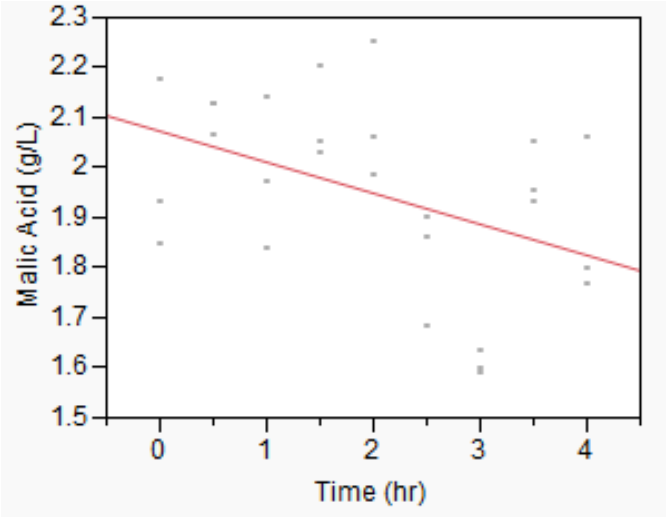


Fig. 6. Malic acid vs. time for MCW 10^7 cells/mL: Malolactic fermentation activity (p=0.002) of resting MCW *O. oeni* at 10^7 cells/ mL in tartrate phosphate buffer at pH 3.5 at 25° C over the course of 4 hours. The rate of fermentation can be described by the equation:

Malic Acid (g/L) = 2.1011023 - 0.0546505*Time (hr)

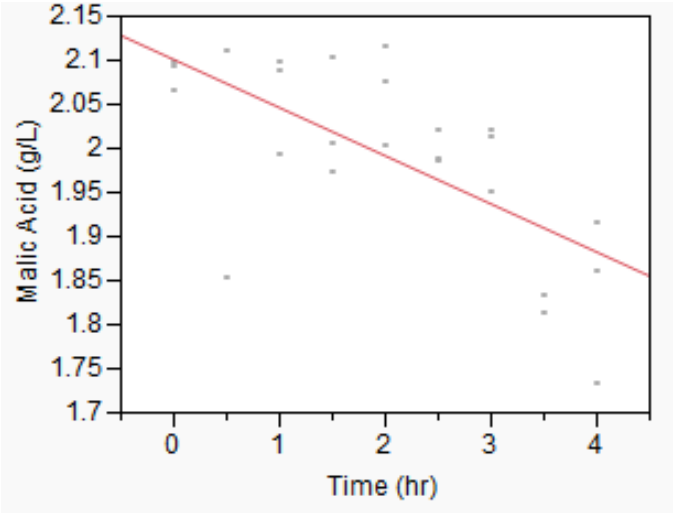
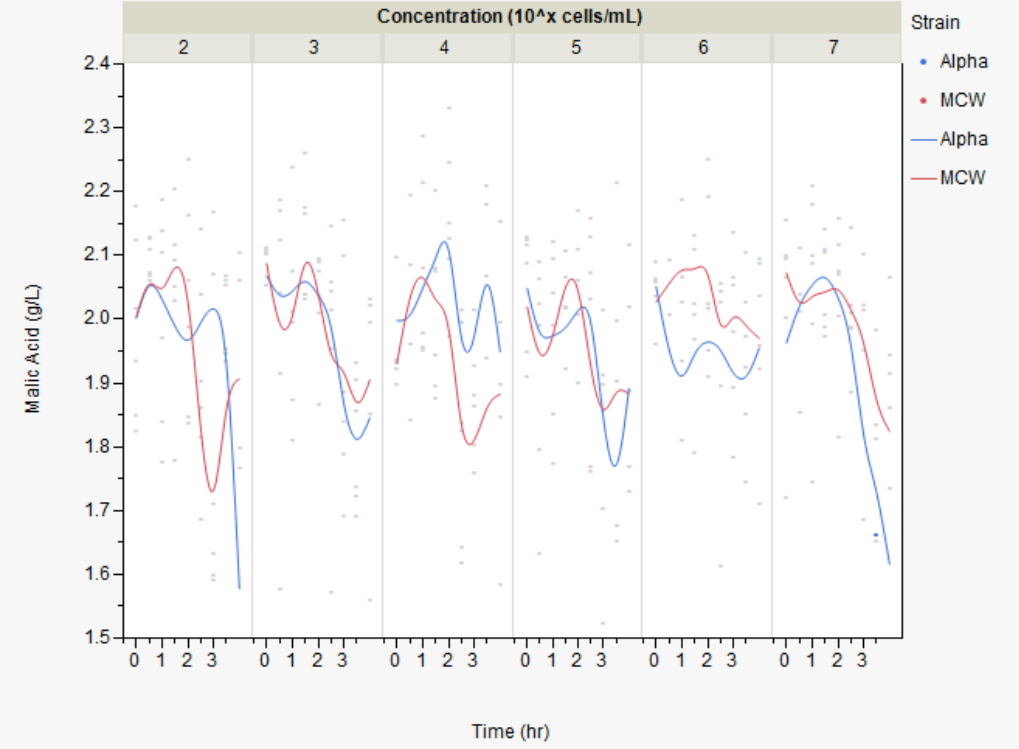


Fig. 7. Malic acid vs. time by concentration and strain: Malolactic fermentation activity of resting Alpha and MCW O. oeni at different concentrations in tartrate phosphate buffer at pH 3.5 at 25° C over the course of 4 hours.



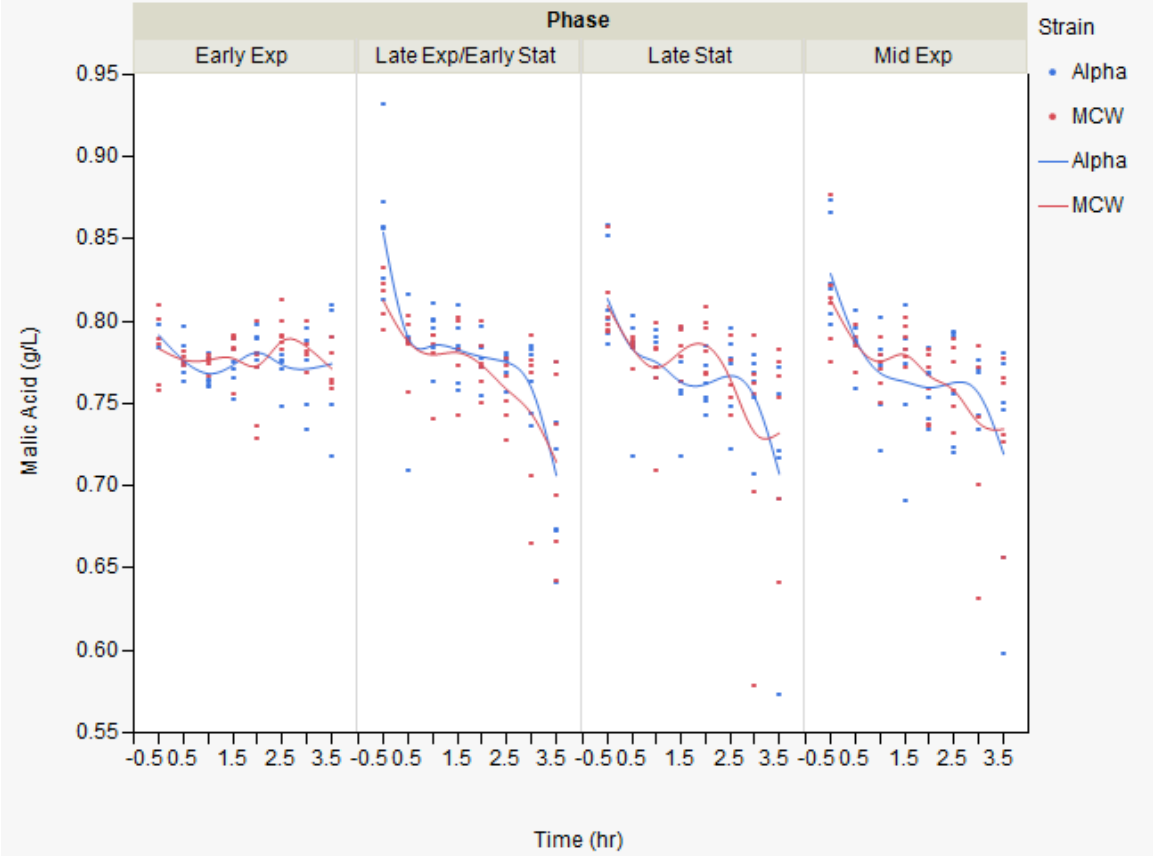
Results for Stage III

The results for Stage III, a study of malolactic fermentation activity at different phases of growth by resting cells of Alpha and MCW *O. oeni*, showed that there was no significant decrease in malic acid over time for either strain at either 10^6 or 10^7 cell/mL in the early exponential phase of growth (Fig. 8). Significant decreases in malic acid over time were observed for both strains at 10^6 and 10^7 cell/mL in the mid exponential, late exponential/early stationary and late stationary phases of growth (Table 1). Rates of malolactic fermentation remained fairly constant for each strain over all of the phases in which malolactic fermentation was performed and did not vary much between strains. Unexplained fluctuations in malic acid concentrations were observed between the first and last measurements. These fluctuations are presumed to be the result of pipetting error during sample collection.

Table 1. Rate of malolactic fermentation: Rate of malolactic fermentation of Alpha and MCW *O. oeni,* harvested at different phases of growth, in tartrate phosphate buffer, at pH 3.5, at 25° C, over the course of 3.5 hours. Significance values are listed in parentheses.

	Rate of malolactic fermentation [(g/L)/hr]			
Phase in the Cell Life Cycle	Alpha 10^6	Alpha 10^7	MCW 10^6	MCW 10^7
Mid Exponential	-0.028(0.0065)	-0.060(0.0004)	-0.038(0.0055)	-0.042(<0.0001)
Late Exponential/Early Stationary	-0.044(0.0051)	-0.070(0.0001)	-0.044(0.0018)	-0.046(0.0001)
Late Stationary	-0.038(0.0008)	-0.048(0.0035)	-0.050(0.0004)	-0.026(0.0053)

Fig. 8. Malic acid vs. time by phase and strain: Malolactic fermentation activity of resting Alpha and MCW *O. oeni,* harvested at different phases of growth, in tartrate phosphate buffer at pH 3.5 at 25° C over the course of 3.5 hours.



Discussion:

Results showed that both strains of *O. oeni* performed malolactic fermentation from the mid exponential to the late stationary phase of population growth. Neither strain performed malolactic fermentation with statistical significance in the early exponential phase during our study. Results also showed that both strains were able to convert malic acid to lactic acid at both low (10^2) and high (10^7) concentrations of cells. Therefore, if there is a concentration of *O. oeni* cells needed for malolactic fermentation to begin, it is below the 100 cells/mL used as the lowest concentration in this study. These results indicate that it isn't the concentration of *O. oeni* that is important, but rather that the metabolism of the cells changes as they enter the mid exponential phase of growth and continues through late stationary phase.

O. oeni ferments grape sugars, when available, as their primary source of carbon for growth (Liu et. al. 1995). No carbon for growth is obtained by *O. oeni* through malolactic fermentation. A possible explanation for the change in malolactic fermentation activity

observed in this study between the early exponential and mid exponential phases of population growth is that malolactic fermentation is a survival mechanism that supplies energy when other nutrients become scarce. Possibly, as *O. oeni* cells begin to run out of some nutrient and move into stationary phase, malolactic fermentation becomes a useful metabolic pathway to supplement or replace sugar catabolism, presumably until better growth conditions occur. Regulation of the malolactic enzyme concentration in *O. oeni*, for instance, may be dependent on stage of growth. It would be interesting to study regulation of malolactic enzyme gene expression under different conditions likely to occur from the mid to late stationary phases of growth, such as low concentrations of sugar, other nutrients, or ATP. It is possible that expression of the malolactic gene does not occur until an energy source better than malic acid is unavailable. The results of this study, showing malolactic fermentation starting during the mid-exponential growth phase, might be explained by this type of regulation. Much work needs to be done to support this speculation, however, and to explain the results seen in this study concerning the effect of the stage of growth on malolactic fermentation activity in *O. oeni*.

Additionally, our results showed that increased rates of malolactic fermentation were not significantly correlated with increased cell concentrations or progression of population growth beyond the mid exponential phase. The lack of correlation between cell concentration and rate of malic acid degradation by *O. oeni* is supported by a recent study (Zinnia et. al. 2011) that derives a mathematical model for malolactic fermentation under ideal conditions. However, this conclusion is contradicted by an earlier study (Maicas et. al. 2000) stating that 10^8 cells/mL is the optimal concentration needed to degrade malic acid in wine. There are a few limitations to this part of the Maicas study that it is important to note. The procedure for determining MLF in wine was not clearly described, but it appears that only one wine was inoculated at 10^8 cells/mL, with no replication performed. This study did not look at inoculation concentrations below 10^7 cells/mL, did not calculate malic acid degradation rates, just measured malic acid concentrations at the beginning and after three weeks, did not monitor cell viability, and examined *O. oeni* malolactic fermentation activity in wine at pH 3.1. This is a stressful environment which may have killed or inhibited many cells, therefore heightening the importance of determining viable cell concentrations in relationship to malolactic fermentation rate.

Lastly, our results suggest that the release of some chemical signal that increases in a large population of *O. oeni* and is sensed once it reaches a sufficient concentration is not what triggers malolactic fermentation, because a threshold concentration of cells necessary for malolactic fermentation to occur was not observed. Therefore, our study suggests that *O. oeni* does not utilize quorum sensing to initiate malolactic fermentation in wines, and probably starts malolactic fermentation under the proper conditions in wines, whether there is a high population of bacteria in the wine or not. To further support this conclusion, the cells were harvested from their original growth medium and washed three times in tartrate phosphate buffer. This preparation would have removed, or at least strongly diluted, any quorum sensing signals from the final cell population. Most of the quorum signals identified for gram positive bacteria, such as lactic acid bacteria, are small peptides, which would be soluble in the aqueous buffer and not replenished by resting cells (Smith et. al. 2004 and Knowings et. al. 2000). Nevertheless,

the presence of quorum sensing signals in the original growth medium was not analyzed in this study, and therefore cannot be ruled out. This experiment needs to be repeated to reduce experimental error to strengthen this conclusion. Additional studies that specifically test for the presence of quorum sensing chemicals need to be completed to rule out their involvement in triggering malolactic fermentation. In addition, the current experiment used resting cells, but experiments using continuous flow bioreactors would be especially helpful to link this study's findings to MLF in wines. This would allow particular concentrations of non-resting cells to be maintained, and media could be continuously flushed to remove, or reduce the concentration of, any quorum-dependent signal chemicals released by the cells (Zhang and Lovitt 2006 and Zinnai et. al. 2011).

While this experiment has provided the first objective laboratory observations regarding the malolactic fermentation activity of *O. oeni* at low cell concentrations and different stages in population growth, it has merely provided the basis for the investigation into the use of quorum sensing by *O.oeni* for malic acid to lactic acid conversion. The occurrence of malic acid conversion under continuous culture and winemaking conditions is still a necessary step toward rejecting quorum sensing as a factor in malolactic fermentation initiation by *O. oeni.*

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