AN ABSTRACT FOR THE THESIS OF

Allison L. O'Neil for the degree of Master of Science in Food Science and Technology presented on August 28, 2009.

Title: Production of SO_2 and SO_2 Binding Compounds by *Saccharomyces cerevisiae* during the Alcoholic Fermentation and the Impact on Wine Lactic Acid Bacteria.

Abstract approved:_____
James P. Osborne

Malolactic fermentation (MLF) is important in the production of wines as it reduces acidity through the conversion of malic acid to lactic acid. However, successful MLF can be difficult to achieve due to yeast antagonism of the bacterium responsible for the secondary fermentation, *Oenococcus oeni*. In particular, yeast produced sulfur dioxide (SO_2) has been implicated in causing problematic MLFs. SO_2 can exist in a free or bound form with the free form thought to have the antimicrobial action. However, some recent studies have demonstrated bacterial inhibition in wine when no free SO_2 was present indicating that bound SO_2 was responsible. Despite this, very little is known regarding the toxicity of the different forms of bound SO_2 and how they may impact the MLF. Therefore, the purpose of this research was to investigate the production of SO_2, and the major SO_2 binding compounds, acetaldehyde, pyruvic acid, and α-ketoglutaric acid, by various commercial wine yeast strains and the impact these compounds have on the MLF.

Fermentations were conducted in a synthetic grape juice and Pinot gris must where viable yeast cell growth, SO_2, acetaldehyde, pyruvic acid, and α-ketoglutaric acid

were measured during the alcoholic fermentation. At weekly intervals samples were taken from the fermentations, sterile filtered, and inoculated with *O. oeni* strain VFO to induce MLF. Progress of MLF was monitored by measuring malic acid and bacterial viable cell counts. Results show that there were significant differences between the amount of SO_2, acetaldehyde, and pyruvic acid produced by the various yeast strains but not α-ketoglutaric acid. Some yeast strains, such as FX10, S102, F15, and M69, produced significantly higher SO_2 concentrations than other yeast strains and *O. oeni* viability decreased rapidly when inoculated into these wines. Very little if any free SO_2 was measured indicating that bound SO_2 and not free SO_2 was responsible for bacterial inhibition. Acetaldehyde bound SO_2 was the dominant species of bound SO_2 found at almost all time points of the alcoholic fermentation indicating that inhibition of MLF by bound SO_2 was due to acetaldehyde bound SO_2.

To further elucidate the role of bound SO_2 in the inhibition of wine lactic acid bacteria (LAB), growth studies on the impact of *O. oeni* strain VFO, *Pediococcus parvulus, P. damnosus*, and *Lactobacillus hilgardii* in media containing free SO_2 or acetaldehyde and pyruvic acid bound SO_2 at two different pHs were performed. In general, inhibition was greater at pH 3.5 than at 3.7 and only *P. damnosus* demonstrated some tolerance to SO_2. Acetaldehyde bound SO_2 appeared to be more inhibitory than either pyruvic acid bound SO_2 or a combination of acetaldehyde and pyruvic acid bound SO_2. Degradation of acetaldehyde appeared to stimulate the growth of *O. oeni* VFO and *Lb. hilgardii* at pH 3.5 but not at 3.7. Overall, a reduction in acetaldehyde and pyruvic acid as well as SO_2 bound acetaldehyde and pyruvic acid was observed for all LAB tested, with the exception of *O. oeni* VFO which did not show a decrease in SO_2 bound

pyruvic acid at pH 3.5. Bacteria were inhibited in media containing acetaldehyde bound SO_2 and pyruvic acid bound SO_2 even though a decrease in SO_2 bound acetaldehyde and pyruvic acid was observed. This suggests that the decrease of the compound bound to SO_2 may have lead to inhibition by the subsequently released free SO_2.

PRODUCTION OF SO_2 AND SO_2 BINDING COMPOUNDS BY *SACCHAROMYCES CEREVISIAE* DURING THE ALCHOLIC FERMENTATION AND THE IMPACT ON WINE LACTIC ACID BACTERIA

by
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A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented August 28, 2009
Commencement June 2010

Master of Science thesis of Allison L. O'Neil presented on August 28, 2009.

APPROVED:

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Dean of the Graduate School

I understand that my thesis will become a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Allison L. O'Neil, Author

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my major advisor, Dr. James P. Osborne, for his support, patience, and guidance during all aspects of my Masters program. I have learned a lot and appreciate the time you spent helping me expand my education here at Oregon State University. I have had a great time getting to know you and your growing family over the past two years.

I would also like to thank Dr. Robert McGorrin and other faculty and staff at the Department of Food Science & Technology for their support during the last few years. I would like to acknowledge Dr. Mark A. Daeschel, Dr. Michael C. Qian, and Dr. Russell E. Ingham for serving on my thesis committee. I would also like to express thanks to Dr. Charles Edwards of Washington State University, Pullman, Washington, USA and ETS Laboratories, St. Helena, California, USA for providing bacterial strains for my research.

I also want to thank David Takush who helped me with my experiments and listened to me talk about how having a thunderstorm in Indiana is not as big a deal as it apparently is in Oregon. I have really enjoyed getting to know you, the game of rugby, and Star Trek. My experience would not have been the same without your friendship.

I would like to express my sincere appreciation to my family, fiancée, and friends for their continual love and support. Specifically my parents who taught me that hard work, kindness, and a belief in GOD are the cornerstones of success. And finally, to my future husband, Justin, thank you for your humorous words of encouragement. I cannot wait for us to not only live in the same time zone but the same town (It only took 4 years and 8 moves but we've done the impossible).

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CHAPTER 1
Introduction

Wine has been an integral part of the human experience for nearly 70 centuries and is closely intertwined with early agriculture, cuisine, and human civilization. Though wine has been made and consumed for millennia, its origin is somewhat disputed. Archeological evidence dating back to around 6,000 BC suggest wine originated in modern day Georgia and Iran (McGovern *et al.*, 2000; Phillips, 2000). Whereas remnants of crushed grapes discovered in Greece dating back to around 6,500 BC suggest winemaking got its beginnings there (McGovern *et al*., 2000; Phillips, 2000). However, wine amphoras were found in King Tutankhamen's tomb and bore the name of the royal vintner. Yet signs of grape skins from Eastern Asia dating back to the first millennium suggest winemaking began there (McGovern *et al.*, 2000). Though the exact origins of wine and winemaking are still unclear, wine is still consumed and enjoyed by humans today.

Wine is produced through the fermentation of grapes by yeast (either naturally found on the grapes or added by the winemaker) that consume the grape sugars and convert them into alcohol and carbon dioxide (CO_2) (Jackson, 2000; Boulton *et al*., 1996; Jackisch, 1985). It is this alcohol that also made drinking wine important in early human civilizations. Acting as an antiseptic, wine was more sterile and "clean" than the local watering hole (McGovern *et al.*, 2000; Phillips, 2000; Vine *et al*., 1997; Boulton *et al.*, 1996). Wine was also able to be stored for long periods of time without spoiling and the ancient Romans used it to prevent dysentery during the expansion of the Roman Empire in 117 AD (McGovern *et al.*, 2000; Phillips, 2000; Vine *et al*., 1997). Wine represents a

safe and healthy beverage providing calories, vitamins, and more importantly a beverage that can bring relaxation and relief from everyday life. It is this great and diverse history of winemaking that has made drinking wine what it is today.

Today, wine is consumed in almost every country in the world and The Wine Institute (2007) estimates that about 20 million acres of land are used for growing grapes, for the winemaking process begins in the vineyard. Grape vines have been cultivated and domesticated for hundreds of years with the vine species *Vitis vinifera* the most commonly used in winemaking today. Grapes of different varieties are cultivated and managed until they reach optimal maturity, usually with a sugar content between 22-25º Brix, with other quality factors considered (Jackson, 2000; Boulton *et al.*, 1996; Fleet, 1992; Jackisch, 1985). Once the grapes have reached ideal maturity they are harvested and sent to the winery to be made into wine.

Upon arriving at the winery, the grapes are typically destemmed and crushed. In white winemaking, the skins are kept in minimal contact with the juice to help reduce the chances of over extracting tannins and other materials that may make the wine astringent (Jackson, 2000; Boulton *et al.*, 1996; Fleet, 1992; Jackisch, 1985). Sulfite in the form of various sulfur salts, such as potassium metabisulfite and sodium metabisulfite, may be added to the must before or after the crush to prevent oxidation and inhibit indigenous microorganisms found on the grapes from growing (Jackson, 2000; Vine, 1997; Jackisch, 1985).

Yeast may then be added to begin the alcoholic fermentation. In some wine regions spontaneous fermentations are still common, however many “newer” winemaking regions, such as South Africa, New Zealand, Australia, and the United

States, predominantly inoculate grape must to induce the alcoholic fermentation (Jackson, 2000; Vine, 1997; Jacksich, 1985). The most commonly used yeast is *Saccharomyces cerevisiae*. *S. cerevisiae* is a vigorous fermentor and has been selected due to its ability to ferment to dryness, its tolerance to high concentrations of ethanol and sulfite, along with producing minimal off-flavors (Jackson, 2000; Boulton *et al.*, 1996; Fleet, 1992; Jackisch, 1985).

Red wines are made in a slightly different manor. After picking, the fruit is crushed and the grape skins and seed are left in contact with the juice, a process called maceration (Jackson, 2000; Boulton *et al*., 1996; Jackisch, 1985). Maceration releases ingredients from the seeds, skins, and pulp and promotes the syntheses of additional flavor compounds. Maceration occurs simultaneously with the alcoholic fermentation and enhances the mouthfeel and texture of a wine (Jackson, 2000; Vine, 1997; Boulton *et al*., 1996; Jackisch, 1985). The alcohol generated by *S. cerevisiae* also enhances the extraction of anthocyanins and promotes the release of tannins from the seed and skins. These phenolic compounds solubilize and give red wines their basic red appearance, taste, and flavor (Jackson, 2000; Vine, 1997; Boulton *et al*., 1996; Jackisch, 1985). At the end of fermentation the wine is pressed off the skins and seeds, racked into barrels, and allowed to age before bottling.

Upon completion of the alcoholic fermentation a second fermentation may be administered, however it is not always necessary. Although not strictly a fermentation, this process is referred to as the malolactic fermentation (MLF) and is performed by a group of wine lactic acid bacteria (LAB), with the species *Oenococcus oeni* being the most common LAB used today. Malic acid is converted to lactic acid and the bacteria use

the malic acid as an energy source for growth (Van Vuuren and Dicks, 1993; Davis *et al.*, 1985; Wibowo *et al.*, 1985; Kunkee, 1967). The major purpose of this process during winemaking is to reduce the acidity of the wine (Fleet, 1992; Kunkee, 1967). MLF usually takes place after the alcoholic fermentation, and occurs at a much slower pace.

At the end of aging, the wine may be filtered and blended with other wines to give them a more distinctive flavor and body. The wine is then bottled and finally shipped to wine shops and grocery stores to be bought by consumers.

CHAPTER 2
Literature Review

Wine fermentation is a complex ecological and biochemical process. Wine has a very low pH, is high in ethanol and organic acids, and lacks most nutrients needed for microbial growth (Jackson, 2000; Fugelsang and Edwards, 2007; Fugelsang, 1997; Fleet, 1992; Jackisch, 1985). It is this harsh environment that allows only a select species of microorganisms to survive and thrive in wine. The extent to which these species grow can determine the types and concentrations of many substances that may contribute to the aroma and flavor of a wine.

The yeast species *Saccharomyces cerevisiae* is the most common wine yeast used in modern winemaking. Even though various microorganisms are commonly found on grapes skins, the vineyard, and winemaking equipment, *S. cerevisiae* tends to dominate as fermentation progresses (Jackson, 2000; Fleet and Heard, 1992). This is primarily due to *Saccharomyces* high alcohol tolerance and ability to grow and ferment under anaerobic conditions.

For all the importance of *S. cerevisiae* in winemaking, grapes are not its original habitat (Jackson, 2000; Naumov, 1996; Martini and Vaughan-Martini, 1990). *S. cerevisiae* is usually absent and rarely found on grapes and little is known as to how it was incorporated into the winemaking process. It is suspected that the surface of winery equipment and the winery itself act as the major source of *S. cerevisiae* (Jackson, 2000; Boulton *et al.*, 1996; Naumov, 1996). However, *Saccharomyces* species possess valuable properties useful in wine production. They typically have the ability to ferment at low temperatures, high pressure, or both, are ethanol, pH, and SO_2 tolerant and produce

minimal off flavors and aromas (Dubourdieu, 2006; Henick-Kling, 2005; Eschenbruch, 1974). *Saccharomyces* species selectively ferment glucose and fructose, can synthesize aromatic compounds, and can impact the mouthfeel of a wine (Dubourdieu, 2006; Boulton *et al.*, 1996, Fleet and Heard, 1992; Jackisch, 1985).

In addition to *S. cerevisiae* certain bacterial species can survive and grow in wine. This includes a number of lactic acid bacteria (LAB). LAB in wine originate primarily from the grapes and winery equipment (Fleet *et al.*, 1984; Costello *et al.*, 1983; Beelman *et al*., 1977; Chalfan *et al.*, 1977). Generally, the organisms occur on the surface of the grapes and vine leaves, at numbers less than 100 cells/g, depending on the maturity and conditions of the berries and vine (Lafon-Lafourcade and Ribereau-Gayon, 1984; Peynaud and Domercq, 1961). Work by Kunkee *et al.* (1965) reported development of a natural malolactic fermentation in a laboratory prepared wine that had not come into contact with winery equipment, indicating that grapes were the source. However, wine equipment, such as storage tanks, pumps, fittings, and valves are also been implicated as sources of LAB (Wibowo *et al*., 1985; Davis *et al*., 1985; Webb and Ingraham, 1960).

The most common LAB present during winemaking is the cocci, obligate heterofermentor *Oenococcus oeni* (formerly *Leuconostoc oenos*) (Cogan and Jordan, 1994; Davis *et al.*, 1985; Wibowo *et al.*, 1985; Garvie, 1980; Beelman *et al*, 1977). This species is typically used to induce malolactic fermentation (MLF), which will be discussed in detail in a later section. *Pediococcus* species and *Lactobacillus* species may also be present but are usually considered to be spoilage bacteria in wine (Henick-Kling, 2006; Carr *et al.*, 2002; Davis *et al.*, 1988). *Lactobacillus* spp. are rod shaped and can be either homo- or hetero-fermentive; whereas *Pediococcus* spp. are strict homofermentators

and are cocci in shape. All three species are gram-positive, non-sporeforming microaerophilic bacteria, are acid and alcohol tolerant, and nutrionally fastidious-requiring amino acids and vitamins for growth (Carr *et al.*, 2002; Van Vuuren and Dicks, 1993; Davis *et al.,* 1985; Wibowo *et al.*, 1985; Garvie, 1980).

LAB may occur and grow at several stages during winemaking. Grape musts soon after crushing generally contain LAB at populations of $1x10^3$ to $1x10^4$ CFU/mL. The major species present at this stage include *O. oeni*, *P. damnosus*, and *Lb. plantarum* (Wibowo *et al.*, 1985; Fleet *et al.*, 1984; Lafon-Lafourcade *et al*., 1983; Kunkee *et al.,* 1965). These species generally do not multiply and die off during the alcoholic fermentation, but a slight increase of some species may occur. By the end of the alcoholic fermentation, the total LAB population has generally declined to a few cells per milliliter (Wibowo *et al.*, 1985; Fleet *et al.*, 1984; Lafon-Lafourcade *et al*., 1983). Sensitivity to ethanol may explain this decline in cell population, but other factors may be operating, since some species, such as *O. oeni* and *P. damnosus,* that die off at this time are able to grow later (Osborne and Edwards, 2005b; Wibowo *et al.*, 1985; Fleet *et al.*, 1984; Lafon-Lafourcade *et al*., 1983).

The depletion of nutrients, such as vitamins or amino acids, by wine yeast may also play a role in the decline of LAB during the alcoholic fermentation (Nygaard and Prahl, 1996; Beelman *et al*., 1982; Fornachon, 1968) as these nutrients are essential for the fastidious LAB. Work performed by Beelman *et al*. (1982) demonstrated that during the alcoholic fermentation, yeast depleted certain amino acids, including arginine, to concentrations that may not have been sufficient for LAB growth. However, after a lag phase, the surviving LAB cells grow and may conduct the MLF. Vigorous bacterial

growth and populations as high as $1x10^6$ to $1x10^8$ CFU/mL characterize this phase (Wibowo *et al.*, 1985; Fleet *et al.*, 1984; Lafon-Lafourcade *et al*., 1983; Kunkee *et al.,* 1965). *O. oeni* is the main species that develops as *O. oeni* usually dominates at low pHs (<3.5), but at high pHs (>3.5) *Pediococcus* spp. and *Lactobacillus* spp. may grow and conduct MLF.

MLF is a secondary fermentation that is commonly practiced in winemaking, particularly during red winemaking. It is preferable induced after alcoholic fermentation but may occur during the alcoholic fermentation (Versari *et al.*, 1999; Markides, 1993). It is the enzymatic decarboxylation of L-malic acid to L(+)-lactic acid and CO_2 (Van Vuurren and Dicks, 1993; Wibowo *et al.*, 1985; Fleet *et al.*, 1984; Lafon-Lafourcade *et al*., 1983; Kunkee, 1967; Kunkee *et al.,*1965). MLF is particularly important for red wines, certain whites, and sparking wines produced in cool climates where the amount of acid in the grape is likely to be quite high (Avedovech *et al.*, 1992; Rodriguez and Amberg, 1990). Deacidification is the most important reason to perform the MLF. The conversion of malic acid (a dicarboxylic acid) to lactic acid (a monocarboxylic acid) reduces the acidity of a wine and causes a corresponding increase in pH (Avedovech, *et al*., 1992; Wibowo *et al.*, 1985; Fleet *et al.*, 1984; Lafon-Lafourcade *et al*., 1983; Kunkee, 1967; Kunkee *et al.,* 1965).

Traditionally the practice relied on natural LAB microflora to induce MLF, but the harsh environment of wine creates a very stressful growth medium for the bacteria and MLF were difficult to conduct. Winemakers overcame this problem by developing select malolactic strains that could perform well in the low pH and high ethanol environment of wine (Alexandre *et al.*, 2004; Van Vuuren and Dicks, 1993; Davis *et al.*,

1985; Wibowo *et al.*, 1985). The most common bacterial species used to conduct MLF is *O. oeni* (Versari *et al.*, 1999; Dicks *et al*., 1995; Davis *et al*., 1985; Wibowo *et al*., 1985) as this bacterium produces minimal off-flavors and aroma and is well suited for growth in wines of low pH (< 3.5) (Versari *et al.*, 1999; Dicks *et al.*, 1995; Davis *et al.*, 1985; Wibowo *et al.*, 1985; Garvie, 1967).

Besides reducing acidity, MLF is believed to influence the microbial stability of the wine, and create desired sensory characteristics. However, there is some dispute regarding the benefits of MLF and its disadvantages (Bartowsky *et al.*, 2002; Lonvaud-Funel, 2002; Henick-Kling, 1993; Wibowo *et al*., 1985; Davis *et al*., 1985). Microbial stability was thought to result from the metabolism of residual nutrients left after alcoholic fermentation by malolactic bacteria (Arnink and Henick-Kling, 2005; Pretorius, 2000; Delcourt *et al*., 1995; Van Vuuren and Dicks, 1993; Davis *et al*., 1985). In addition, the complex nutrient demands for the LAB were thought to reduce the concentrations of amino acids, nitrogen bases, and vitamins thus creating an environment very difficult for any microorganism to survive in. However, although levels of nutrients may decrease during MLF, this is not a consistent finding. Vetsch and Mayer (1978) reported the growth of *P. damnosus* to levels of $1x10^7$ CFU/mL in a red wine (pH 3.8 to 3.9) after MLF. Costello *et al.* (1983) also observed the growth of *Lactobacillus* spp. and *Pediococcus* spp. in Australian Shiraz wines (pH 3.69) once MLF had been completed by *O. oeni.*

MLF may also contribute to the sensory qualities of a wine by the production of many flavor and aroma compounds (Alexandre *et al.,* 2004; Jackson, 2000; Henick-Kling 1993; Van Vuuren and Dicks, 1993; Davis *et al*., 1985; Kunkee, 1967). Changes in flavor

compounds, such as acetaldehyde, acetic acid, acetoin, and diacetyl (2,3-butanedione), have been measured in wine post MLF (Martineau and Henick-Kling, 1995; Van Vuuren and Dicks, 1993; Rodriguez and Amberg, 1990; Collins, 1972; Fornachon and Lloyd, 1965). Of the mentioned, diacetyl is the most important. It has a distinct buttery aroma that is synthesized from citrate or other carbohydrates (Lui, 2003; Martineau and Henick-Kling, 1995). The presence of diacetyl at low concentrations (1 to 3 mg/L) is described as "buttery" or "nutty", concentrations exceeding this level (5 to 7 mg/L) will dominate the wine, and is regarded by some as a defect (Wibowo *et al*., 1985; Rankine *et al.,* 1969). *O. oeni* has also been shown to produce certain esters, such as ethyl acetate and ethyl lactate, which may impact wine aroma (Delaquis-Pascal *et al*., 2000; De Revel *et al*., 1999; Maicas *et al*., 1999; Edwards and Peterson, 1994).

Many researchers have reported that the ability of *O. oeni* to successfully conduct the MLF is impacted by the yeast used to conduct the alcoholic fermentation (Nygaard and Prahl, 1996; Henschke and Jiranek, 1993; Fourcassier *et al*., 1992; Guilloux-Benatier *et al*., 1985; Fornachon, 1968). This interaction may be stimulatory (Henschke and Jiranek, 1993; Fourcassier *et al*., 1992; Fornachon, 1968) or inhibitory (Osborne and Edwards, 2006; Larsen *et al,* 2003; Nygaard and Prahl, 1996; Henick-Kling and Park, 1994; Henick-Kling, 1993; Edwards and Beelman, 1987; Lonvaud-Funel *et al*., 1988). For example, early work by Fornachon (1968) recognized that in addition to antagonism of yeast, some yeast strains produced wine that was more favorable towards the growth of LAB. Interestingly, it was also observed that the antagonism of yeast towards bacteria was generally reduced if wines were left in contact with yeast lees after fermentation. Fornachon (1968) suggested this phenomenon, which varied by yeast strain, might have

been associated with the rate of yeast autolysis. It is generally recognized that substrates released by yeast, particularly nitrogenous compounds, may play a role in influencing bacterial growth in wine (Henschke and Jiranek, 1993; Fourcassier *et al*., 1992; Feuillat *et al*., 1985). Work performed by Guilloux-Benatier *et al*. (1985) showed that yeast autolysates prepared with different levels of proteolysis stimulated the growth of malolactic activity of different LAB. However, the knowledge of the specific nitrogen components derived from yeast that can be utilized to stimulate LAB is limited (Fourcassier *et al.*, 1992). In addition, yeast mannoproteins, which constitute a major component of the macromolecules produced by yeast, have also been associated with stimulation of bacterial growth in wine (Guilloux-Benatier *et al.*, 1985). It has been proposed that the mannoproteins released during the alcoholic fermentation or autolysis may adsorb medium chain fatty acids synthesized by *Saccharomyces* that are inhibitory to *O. oeni* (Guilloux-Benatier and Feuillat, 1991).

Contrary to the stimulation of MLF, the growth of yeast during the alcoholic fermentation has been shown to inhibit MLF and the growth of *O. oeni*. Although the biochemical basis for negative yeast/bacteria interactions are still unclear, some researchers have suggested that removal of nutrients by the faster growing yeast is responsible for MLF inhibition (Lafon-Lafourcade, 1984; Ribéreau-Gayon *et al*., 1975; Rankine *et al*., 1970; Amerine and Kunkee, 1968). For example, Beelman *et al.* (1982) demonstrated that during fermentation with a pure culture certain amino acids, especially arginine, were depleted to concentrations that may not support MLF. Nevertheless, leaving wine in contact with yeast lees can replenish amino acids and other nutrients through passive release and yeast autolysis and may affect the proliferation of MLF

(Pretchard and Coolbear, 1993; Gullioux-Benatier and Feuillat, 1991; Gullioux-Benatier *et al.*, 1985; Fornachon, 1968).

In addition, production of antibacterial proteins by yeast has also been reported (Osborne and Edwards, 2007; Capucho and San Ramao, 1994; Dick *et al.* 1992). In a study performed by Osborne and Edwards (2007), the growth of *O. oeni* and the MLF was inhibited by the yeast RUBY.ferm but not inhibited by EC1118 despite similar amounts of total sulfur dioxide (SO_2) produced by the two yeast strains. As such, the inhibition of MLF by *S. cerevisiae* RUBY.ferm appeared to be due to factors other than the production of SO_2. Using SDS PAGE, Osborne and Edwards (2007) identified a possible 5.9 kDa antibacterial peptide from the wine fermented with yeast RUBY.ferm. This is a consistent finding with Dick *et al.* (1992) who reported two possible antibacterial protein(s) or peptide(s) produced by *S. cerevisiae* shown to inhibit LAB.

Yeast produced medium chain fatty acids, such as decanoic acid, have also been shown to inhibit both yeast and wine bacteria (Versari *et al*., 1999; Edwards and Beelman 1987). In addition to limiting bacterial growth, medium chain fatty acids can reduce the ability of LAB to decarboxylate L-malic acid although this is highly dependent on the type and concentration of the fatty acid in the medium (Versari *et al*., 1999). Edwards and Beelman (1987) demonstrated that the addition of 5 to 10 mg/L decanoic acid to grape juice suppressed bacterial growth, where as 30 mg/L was lethal and completely inhibited the MLF. Consequently, Capucho and San Romao (1994) reported that decanoic and dodecanoic acids at concentrations below 12.5 and 2.5 mg/L, respectively, were stimulatory to malolactic activity; whereas higher concentrations of the two acids became inhibitory to both the malolactic enzyme and bacterial growth. Finally, Lonvaud-Funel *et*

al. (1988) indicated that when a mixture of fatty acids was added to the medium the bacterial growth was inhibited greater than the addition of the individual acid.

Overall it is apparent that a number of different yeast derived substances can exert inhibitory effects towards LAB bacteria. However, the addition of and the ability for yeast to produce SO_2 is frequently associated to bacterial inhibition (Larsen *et al*., 2003; Osborne *et al*., 2000; Henick-Kling and Park, 1994; Hood, 1983: Fornachon, 1963). SO_2 is commonly added by winemakers to control oxidation and growth of undesirable spoilage microorganisms (Jackson, 2000; Boulton *et al*., 1996; Jackisch, 1985) but may also be produced by yeast. In an aqueous solution, SO_2 exists in an equilibrium between molecular gas ($SO_2 \bullet H_2O$), bisulfite ion (HSO_3^-), and the sulfite anion (SO_3^{2-}) species (Swiegers and Pretorius, 2007; Rose and Pilkington, 1989; Hinze and Holzer, 1986; Eschenbruch, 1974; Hammond and Carr, 1976), as illustrated below:

$$SO_2 + H_2O \leftrightarrows SO_2 \bullet H_2O$$
$$SO_2 \bullet H_2O \leftrightarrows HSO_3^- + H^+$$
$$HSO_3^- \leftrightarrows SO_3^{2-} + H^+$$

The proportions of these species found in solution are dependent on the wine pH, with the dominant species at wine pH (3 to 4) being the bisulfite ion (Figure 2.1).

Winemakers add SO_2 to wine for a number of reasons. One major reason is to prevent oxidation of the wine (Li *et al*., 2008; Jackson, 2000; Vangarde and Woodburn, 1994). Oxidative reactions can occur in both red and white wines, but are particularly noticeable in the latter. Dissolved oxygen in wine can react with phenol compounds giving the wine a brownish hue (Li *et al*., 2008; Vangarde and Woodburn, 1994). SO_2 is used by winemakers to prevent this oxidation however it does not act by directly

removing oxygen from wines and musts; rather SO_2 prevents oxidation by binding with the precursors involved in the oxidative reactions preventing them from reacting with oxygen or by binding with compounds already oxidized to reverse the effect of oxygen. SO_2 also acts by reducing the activity of the polyphenol oxidase enzyme, another cause of browning in white wines (Li *et al*., 2008; Gould, 1996; Vangarde and Woodburn, 1994; Jackisch, 1985).

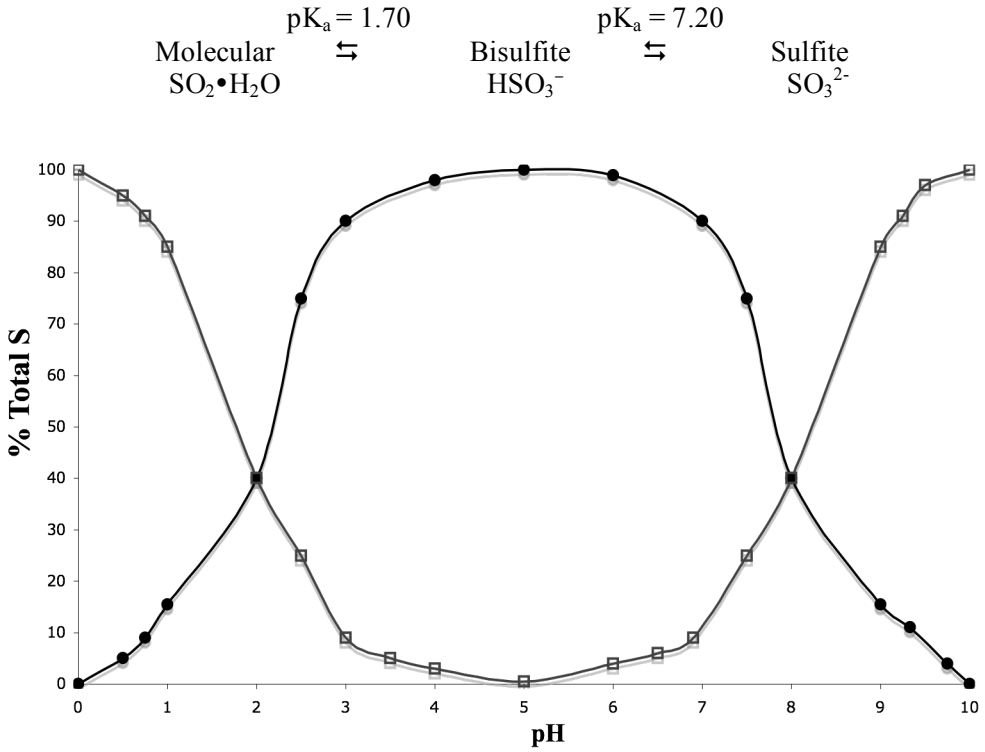


Figure 2.1: Relative concentrations of molecular SO_2, bisulfite, and sulfite at different pH values (Adapted from Fugelsang and Edwards, 2007).

In addition SO_2 is also added during winemaking to control the growth of undesirable yeast, molds, and bacteria. For antimicrobial activity, the free form of SO_2 is believed to be most effective, with the molecular SO_2 form being the most antimicrobial. This form can enter the cell and inhibit growth or cause cell death at levels of around 0.5 to 1 mg/L (Jackson, 2000; Jarvis and Lea, 2000). Work performed by Macris and

Markakis (1974) showed that molecular SO_2 is the most readily absorbed and toxic to microorganisms. Strantford *et al*. (1987) confirmed Macris and Markakis' finding and determined that the most inhibition/cell death was when the pH ranges from 3 to 5. They suggest that molecular SO_2 is transported into *S. cerevisiae* by simple diffusion through the cell membrane, a finding later supported by multiple researchers (Rose, 1993; Rose and Pilkington, 1989; Taylor *et al.*, 1986; Maier *et al.*, 1985). Although Donalies and Stahl (2002) have suggested SO_2 enters the cell via an active carrier mediated process.

Once inside the cell, molecular SO_2 dissociates, delivering hydrogen ions along with the sulfite anions into the cells cytoplasm (Donalies and Stahl, 2002; Stratford *et al*., 1987; Macris and Markakis, 1974). The additional hydrogen ions may be exported to maintain a high internal pH, but this is energy demanding, so cell growth is restricted. If energy demand is overcome, the pH of the cytoplasm eventually falls to a level that is too low for growth to continue (Donalies and Stahl, 2002; Gould, 1996; Stratford *et al*., 1987; Macris and Markakis, 1974). Intracellular SO_2 also disrupts protein disulfide bonds that are needed for many enzymes and regulatory proteins in the cell (Rose, 1993; Rose and Pilkington, 1989; Taylor *et al*., 1986) and the binding of SO_2 with nucleic acids and lipids can cause genetic and membrane dysfunction, respectively. Additional, antimicrobial activity may also be attributed to a rapid drop in the intracellular pool of ATP and NAD^+/NADH due to the disruption of the glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase and alcohol dehydrogenase, respectively (Rose, 1993; Rose and Pilkington, 1989; Taylor *et al*., 1986; Hinze and Holzer, 1986; Maier *et al*., 1985).

Bacterial sensitivity to SO_2 has been widely reported (Larsen *et al*., 2003; Osborne *et al.,* 2000; Eglinton and Henschke, 1996; Zoecklein *et al,* 1995; King and

Beelman, 1986) with Gram negative bacteria generally being more sensitive to SO_2 than Gram positive bacteria (Gould, 1996; Hammond and Carr, 1976). Although there is considerable strain variation in the response of wine LAB to SO_2, it seems that species of *Lactobacillus* and *Pediococcus* are more tolerant of SO_2 than *O. oeni* (Davis *et al*., 1988; Hood, 1983; Carr *et al*., 1976; Lafon-Lafourcade and Peynaud, 1974; Fornachon, 1963). For example, in a study by Davis *et al*. (1988) bacterial strains *O. oeni*, *P. parvulus*, and *Lactobacillus* spp. were grown in a nutrient medium, pH 4.5, containing 64 mg/L of total SO_2. When the total SO_2 concentration was increased to 128 mg/L only 11% of the *O. oeni* strain grew, where as 74% of the *Pediococcus* spp. and 59% of the *Lactobacillus* spp. tested grew. Overall, information on the effects of SO_2 on specific species of wine LAB is vague and clarification is required.

Although SO_2 is toxic to wine microorganisms, yeast produce it in various concentrations during alcoholic fermentation. The ability of *S. cerevisiae* wine yeast to produce SO_2, via the reductive sulfate assimilation pathway (APS), is dependent upon various factors, including the strain involved, fermentation temperature, and the wines composition (Jarvis and Lea, 2000; Henick-Kling, 1994; Eschenbruch, 1974; Weeks, 1969). Most yeast strains produce < 30 mg/L total SO_2 although some have been reported to produce >100 mg/L (Osborne and Edwards, 2005; Jarvis and Lea, 2000; Henick-Kling, 1994; Eschenbruch, 1974; Weeks, 1969). The process to assimilate sulfate is both complex and expensive in the use of ATP and NADPH. Simply, extracellular sulfate (SO_4^{2-}) is transferred across the cell membrane, transformed, and reduced to its final electron donor, S-Adenosyl-Methionine (AdoMet) which can then be used for various methylation reactions throughout the cell or incorporated in the biosynthesis of the sulfur

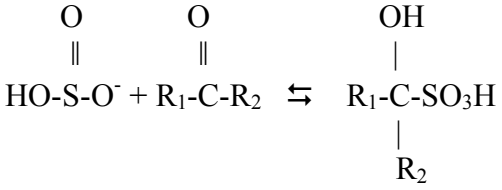
containing amino acids cysteine and methionine (Roje, 2006; Thomas and Sudrin-Kerjan, 1997).

The addition of SO_2 to grape must in conjunction with yeast produced SO_2 may result in the wine being inhibitory to the growth of LAB. For example, Henick-Kling and Park (1994) suggested that the concentration of SO_2 added to grape juice, combined with that produced by the yeast strain during alcoholic fermentation was a major factor in determining the success of LAB starter cultures. Henick-Kling and Park (1994) further suggested that yeast produced SO_2 is a primary mechanism for bacterial inhibition.

Although free SO_2 (and in particular the molecular portion) is considered to be the most antimicrobial form of SO_2 (Rose and Pilkington, 1989; Hinze and Holzer, 1986; Eschenbruch, 1974; Hammond and Carr, 1976), a number of researchers have suggested that bound SO_2 may be more antimicrobial than previously believed (Larsen *et al*., 2003; Henick-Kling and Park 1994; Hood, 1983; Fornachon, 1963). For example, Fornachon (1963) found that MLF was prevented in the presence of bound SO_2 where no free SO_2 was measurable. More recently, Larsen *et al*. (2003) and Osborne and Edwards (2006) found a strong correlation between yeast production of SO_2 and the inhibition of *O. oeni* in both synthetic media and in grape juice. These authors reported that all SO_2 measured was present in unidentified bound forms as free SO_2 was not detected either by titration or capillary electrophoresis analysis. These finding suggests that bound forms of SO_2 could be much more antibacterial than previously thought.

Bound SO_2 occurs when in an aqueous environment SO_2 reacts with carbonyl compounds (e.g. acetaldehyde), forming adducts called hydroxysulfonic acids. Binding compounds can naturally be found in grape must, however a majority of the binding

compounds in wine are produced by yeast during the alcoholic fermentation (Azevedo *et al*., 2007; Jackson, 2000; Jackisch, 1985). The three major SO_2 binding compounds present in wine include acetaldehyde, pyruvic acid, and α-ketoglutaric acid, with acetaldehyde having the highest affinity for SO_2, followed by pyruvic acid and α-ketoglutaric acid (Larsen *et al.*, 2003; Burroughs and Sparks, 1973; Rankine and Pocock, 1969; Fornachon, 1963). The concentration of these three binding compounds in wine account for between 59 to 77% of bound SO_2 found in wine (Rankine, 1969). Other compounds, such as sugars and anthocyanins, also have the ability to bind to SO_2 but these are weakly bound and are not as significant as the above three mentioned (Burroughs and Sparks, 1973). Still, all SO_2 binding substances contain either one or two carbonyl groups to form covalently bound carbonyl bisulphate compounds (hydroxysulfonic acids) (Azevedo *et al.,* 2007; Burroughs and Sparks, 1973). A general reaction is illustrated below:



The concentration of each individual binding compound in the medium varies, and is based on the yeast strain, pH, fermentation temperatures, and total SO_2 level (Larsen *et al.*, 2003; Zoecklein *et al*., 1995; Burroughs and Sparks, 1973; Rankine and Pocock, 1969; Rankine, 1968; Lafon-Lafourcade and Peynaud, 1966; Fornachon, 1963). The equilibrium between free and bound SO_2 can move in either direction if the concentration of one of the reactants is altered (Azevedo *et al*., 2007; Burroughs and

Sparks, 1973). Thus if more SO_2 is added or the pH is changed, part of the SO_2 will become bound or free, and conversely, if SO_2 is removed binding compounds will be liberated by the dissociation of carbonyl bisulphate.

In addition, free SO_2 is also in equilibrium between each individual carbonyl compound due to the Law of Mass Action (Burroughs and Sparks, 1973). Therefore, equilibrium constants (K) unique to each carbonyl compounds have been determined; with small K values indicate a tighter binding and vice versa (Azevedo *et al*., 2007; Jarvis and Lea, 2000; Burroughs and Sparks, 1973). Thus, although there is typically more glucose in wine than acetaldehyde, acetaldehyde ($K=1.5x10^{-6}$) makes a much greater contribution to SO_2 binding than glucose ($K=6.4x10^{-1}$). It is also important to note that the binding power of acetaldehyde is so great that, in practice, no free SO_2 can exist in a wine until all the acetaldehyde is bound.

Acetaldehyde is considered to be a leakage product of alcoholic fermentation by yeast and excreted mainly during the yeast growth phase (Martinez *et al*., 1997; Margalith, 1981; Amerine and Ough, 1964; Weeks, 1969; Ribereau-Gayon *et al,* 1956). Acetaldehyde is produced as an intermediate in the reduction of pyruvic acid to ethanol by reacting with NAD^+ and the enzyme acetaldehyde dehydrogenase, refer to Figure 2.2 (Berg *et al.,* 2007; Liu and Pilone, 2000).

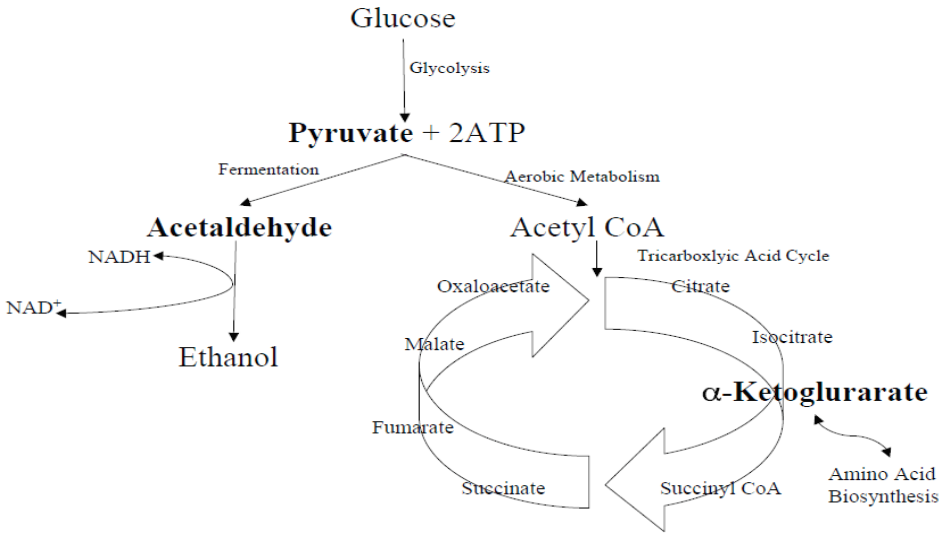


Figure 2.2: Simplified biochemical pathway for the production of pyruvic acid, acetaldehyde, and α-ketoglutarate by *S. cerevisiae*

Although fermentation is the primary biochemical process for acetaldehyde production, it may also be produced through the metabolism of certain amino acids, such as alanine (Berg *et al*., 2007; Boulton *et al*., 1996; Henschke and Jiranek, 1993).

It addition, acetaldehyde can be formed from coupled auto-oxidation (Wildenradt and Singleton, 1974). Direct oxidation of ethanol is insignificant, but the oxidation of ethanol to acetaldehyde can occur by a coupled auto-oxidation of certain phenolic compounds (Lui and Pilone, 2000; Wildenradt and Singleton, 1974). This is caused by the actions of a strong oxidant, such as hydrogen peroxide (produced during phenolic oxidation), which then oxidizes ethanol to acetaldehyde. Acetaldehyde is also formed from the oxidation of ethanol by film yeast in Sherry making (Fugelsang, 1997; Zoecklein *et al*., 1995; Sponholz, 1993). Acetaldehyde is the most important sensory

compound formed during Sherry making and constitutes of more than 90% of the total aldehyde content in Sherry wine (Lui and Pilone, 2000). While at low levels, acetaldehyde gives a pleasant fruity aroma, at higher levels above its sensory threshold of 100 to 125 mg/L, acetaldehyde tends to create a green, grassy or apple-like aroma (Lui and Pilone, 2000; Jackson, 2000; Adams and Moss, 1995; Miyake and Shibamoto, 1993).

Various levels of acetaldehyde are found in wine with average values of about 80 mg/L for white wine, 30 mg/L for red wine, and 300 mg/L for Sherries (Lui and Pilone, 2000; Zoecklein *et al,* 1995; Williams, 1970). External factors such as temperature, oxygen, and SO_2 can affect the production of acetaldehyde by yeast (Ough, 1985). The use of SO_2 has also been suggested to induce acetaldehyde formation by yeasts (Henick-Kling *et al*., 1998; Romano *et al*., 1994; Pilkington and Rose, 1988; Stratford *et al*., 1987; Rankine and Pocock, 1969; Weeks, 1969) and wines fermented with SO_2 have been shown to have higher acetaldehyde levels than wines made without SO_2 (Henick-Kling *et al*., 1998; Herraiz *et al*., 1989). This SO_2 induced production of acetaldehyde is suggested to be related to yeasts resistance to SO_2 (Pilkington and Rose, 1988; Stratford *et al*., 1987) however further research on this topic is needed. The addition of SO_2 to wine can also mask the sensory impact of acetaldehyde due to acetaldehydes high affinity for SO_2, thus protecting and/or improving the wines taste or aroma (Liu and Pilone, 2000). However, addition of high levels of SO_2 can affect the polymerization reaction between acetaldehyde and wine phenolics, which have a decolorization effect (Ribereau-Gayon *et al*., 1983).

Compared to yeast, there has been no definitive study on the effect of acetaldehyde on wine LAB, although a number of studies have reported the ability of

wine LAB to degrade acetaldehyde (Osborne *et al*., 2000; El-Gendy *et al*., 1983; Hood, 1983; Collins and Speckman, 1974; Fornachon, 1963). For example, Osborne *et al.* (2000) reported *O. oeni* and *Lactobacillus* spp. could degrade acetaldehyde to ethanol and acetic acid while two *Pediococcus* spp. could not. Other researchers also reported the ability of some wine LAB especially heterofermenters, such as *O. oeni,* to catabolize acetaldehyde during fermentation (El-Gendy *et al*., 1983; Collins and Speckman, 1974). Although the reason for this is not completely understood, one thought by Osborne *et al.* (2000) is acetaldehyde metabolism can act as a NAD^+/NADH generator that can help the bacterium to regenerate electrons in fermentation. Whereas, Collins and Speckman (1974) and Lindsay *et al*. (1965) suggest that acetaldehyde acts as a hydrogen acceptor during heterofermentation with the formation of extra energy (ATP), which stimulates growth.

After acetaldehyde, the next most important SO_2 binding compound produced by yeast during fermentation is pyruvic acid. Pyruvic acid plays a central role in energy metabolism in living organisms (Romano *et al*., 1994; Whiting and Coggins, 1973). Referring to Figure 2.2 carbohydrates, notably glucose, are processed by glycolysis into pyruvate. One molecule of glucose is catabolized into two molecules of pyruvate, which are then used to provide further energy in a variety of other biochemical pathways (Berg *et al.*, 2007; Liu, 2003; Axelsson, 1998; Romano *et al*., 1994; Kandler, 1983; Whiting and Coggins, 1973). Under aerobic conditions, pyruvate is oxidatively decarboxylated by the pyruvate dehydrogenase complex to form acetyl coenzyme A (acetyl CoA) (Berg *et al*., 2007). This is an irreversible reaction and is the link between glycolysis and the tricarboxylic acid cycle (TCA) (Berg *et al.*, 2007; Axelsson, 1998; Kandler, 1983). On

the other hand, in anaerobic conditions, pyruvic acid is converted into ethanol. Pyruvic acid is decarboxylated by pyruvate decarboxylates in the presence of the coenzyme thiamine pyrophosphate to generate acetaldehyde, which is later decarboxylated again to form ethanol (Berg *et al.*, 2007; Axelsson, 1998; Kandler, 1983).

Pyruvate can also be produced directly or indirectly from amino acids by transamination (Liu, 2003, Liu *et al*., 1995; Chen *et al*., 1983; Carr and Davies, 1970; Weimer *et al,* 1999). For example, pyruvate is formed directly from alanine via transamination where alanine plus an amino acceptor (α-ketoglutarate) (Liu, 2003; Berg *et al.*, 2007; Chistensen *et al*., 1999; Weimer *et al*., 1999). In addition, some amino acids directly or indirectly derived from pyruvate can also affect the metabolic flux of pyruvate in yeast and bacteria (Liu, 2003).

As pyruvate is located at a key junction of metabolism (Figure 2.2), only a small amount of pyruvic acid is usually secreted into the media during normal sugar metabolism by microorganisms (Wang *et al*., 2002). Under certain conditions, such as reduced pH and oxygen, excessive pyruvic acid production has been observed from bacteria (Yokota *et al*., 1994; Yokota *et al*, 1989) and yeast (Yonehara and Miyata, 1994; Moriguchi *et al*., 1984). Yonehara *et al*. (2000) have shown that many vitamins needed for the pyruvate dehydrogenase coenzyme can also affect pyruvate production in yeast.

Apart from pyruvate production during fermentation microorganisms can also consume it. LAB can metabolize pyruvate to produce lactate (Liu, 2003) along with other compounds, such as ethanol, acetaldehyde, diacetyl, acetic acid, and 2,3-butanediol (Axelsson, 1998; Kandler, 1983; Radler, 1975; Pilone and Kunkee, 1972). For example, diacetyl is formed by *O. oeni* during MLF via the reductive decarboxylation of pyruvic

acid to 2,3-butanediol (Martineau and Henick-Kling, 1995). However, to date, microbial stimulation by pyruvate has not been observed.

The amount of pyruvic acid produced by yeast varies considerably. Deibner and Hugues (1966) found, on average, 63.6 mg/L of pyruvic acid where other researchers have found it to range between 11 to 400 mg/L (Lafon-Lafourcade and Peynaud, 1966, Rankine, 1965). Differences in the average production of pyruvic acid are dependent of yeast strain, pH (Rankine, 1967), grape variety (Rankine and Pocock, 1969), nitrogen source, and thiamine deficiency (Rankine, 1965).

The third most important SO_2 binding compound in wine is α-ketoglutaric acid. In wine yeast, α-ketoglutaric acid is mainly formed as a deanimation product from glutamic acid in connection to protein biosynthesis (Figure 2.2) (Berg *et al.*, 2007). It is also one of the main constituents of the TCA cycle in microorganism, but since this cycle does not operate in anaerobic fermentation, it would not function as a step in energy metabolism (Berg *et al*., 2007; Jackson, 2000; Fleet, 1992). The influence of yeast strains on the formation of α-ketoglutaric acid formation has not been well studied. However, work performed by Rankine (1968) reported production of α-ketoglutaric acid by twelve *Saccharomyces* strains and showed production between 9 to 117 mg/L by the various strains. The amount of α-ketoglutaric acid normally present in wine is unlikely to have any direct effect on the aroma and flavor of wine, but may significantly affect the binding of SO_2.

Because wine yeast strains can produce different concentrations of the major SO_2 binding compounds, various concentrations of acetaldehyde bound SO_2, pyruvic acid bound SO_2, and α-ketoglutarate bound SO_2 will be present in a wine depending on what

yeast conducts the alcoholic fermentation. However, the effect of the production of SO_2 binding compounds on the forms of bound SO_2 present in wine and the toxicity of these various forms to malolactic bacteria has not been investigated. In fact, the toxicity of bound SO_2 to wine bacteria is still not well understood. While a number of researchers have shown that high concentrations of total SO_2 inhibited MLF (Arnick and Henick-Kling, 2005; Markides, 1993; Hammond and Carr, 1976), others reported that MLF was inhibited by SO_2 when the amount of measured free SO_2 was practically nil (Osborne and Edwards, 2006; Larsen *et al*., 2003; Henick-Kling and Park, 1994; Eglinton and Henschke, 1996, King and Beelman, 1986; Fornachon, 1963).

Fornachon (1963) and Larsen *et al*. (2003) reported inhibition of MLF by yeast produced SO_2 where unidentified bound forms of SO_2 were found in large amounts while free SO_2 was not detected by either titration or capillary electrophoresis. In addition, a study conducted by Mayer *et al*. (1976) saw similar MLF inhibition as Fornachon (1963) and found an inverse relationship between bacterial growth and the level of bound SO_2 remaining after fermentation by various yeast strains. Mayer *et al*. (1976) concluded that when more than 50 mg/L bound SO_2 remained after the primary fermentation bacterial growth and MLF were prevented. It is apparent that bound SO_2 possess some antibacterial properties, although very little experimental evidence shows exactly which form(s) of yeast produced bound SO_2 inhibits the growth of bacteria in wine and the mechanism for LAB inhibition (Eglintion and Henschke 1996; Fornachon 1963).

There is conflicting information as to the mechanism of MLF inhibition to wine bacteria. The argument as to whether inhibition is caused directly by bound SO_2 or due to the release of the SO_2 moiety in bound SO_2 by LAB has not produced definitive results.

Early work by Fornachon (1963) reported that both *Lactobacillus hilgardii* and *Leuconostoc mesenteriodes* were inhibited in a medium in which sulfurous acid and an excess of acetaldehyde had been added, suggested that these bacteria could metabolize acetaldehyde bound SO_2, an observation later confirmed for *O. oeni* (Osborne *et al*., 2006). In spite of this, Carr *et al*., (1976) reported that acetaldehyde bound SO_2 did not have an influence on the bacterium *Lactobacillus plantarum* or on MLF.

Hood (1983) provided an alternative mechanism by suggesting that any effect of bound SO_2 was due to small amounts of free (specifically molecular) SO_2 in equilibrium with the bound form and also suggested that pyruvic acid bound SO_2 has a greater effect on bacterial growth than acetaldehyde bound SO_2. Work by Larsen *et al*. (2003) helped support Hoods theory of compounds other than acetaldehyde bound SO_2 causing MLF inhibition. Larsen *et al*. (2003) showed that *O. oeni* was not inhibited in fermentations induced by yeast strains containing similar molar concentration of bound SO_2 and acetaldehyde; whereas strong inhibition was observed in fermentations containing a much higher proportion of SO_2 bound to unidentified molecules other than acetaldehyde.

In spite of the fact that a number of researchers have investigated the effects of bound SO_2, with most of the focus being on acetaldehyde bound SO_2 (Larsen *et al*., 2003; Osborne *et al*., 2000; Hood 1983; Rankine 1965; Fornachon, 1963) little research has been undertaken investigating the affects of pyruvic acid and α-ketoglutarate bound SO_2 on the MLF and LAB (Larsen *et al*., 2003). If different yeast strains produce different amounts of SO_2 binding compounds this may have to be accounted for when considering yeast/bacterial combinations and the role bound SO_2 plays in causing problematic MLFs may need to be considered. Knowledge regarding the production of SO_2 binding

compounds by yeast may also minimize SO_2 use through the choice of yeast strain based on the production of SO_2 binding compounds. For example, use of yeast strains that produce low amounts of binding compounds may allow the winemaker to add less SO_2 to maintain an effective level for antimicrobial purposes. This reduction of SO_2 use is beneficial, as there is mounting consumer resistance to the excessive use of SO_2 and other chemical preservatives in wine (Du Toit and Pretorious, 2000). Therefore the objective of this study was to investigate the ability of yeast strains to produce SO_2 and SO_2 binding compounds and the impact of these compounds on the MLF and common wine LAB.

CHAPTER 3

Production of SO_2 and SO_2 binding compounds by *Saccharomyces cerevisiae* during alcoholic fermentation and the impact on malolactic fermentation

ABSTRACT

The objective of this study was to investigate the ability of *Saccharomyces cerevisiae* to produce sulfur dioxide (SO_2) and SO_2 binding compounds and the impact these compounds have on the malolactic fermentation (MLF). Fermentations were conducted in both a synthetic grape juice and a Pinot gris juice and SO_2, acetaldehyde, pyruvic acid, and α-ketoglutaric acid were measured at multiple times during the fermentation. At weekly intervals samples were taken from the fermentations, sterile filtered, and inoculated with *O. oeni* strain VFO to induce MLF. Progress of MLF was monitored by measuring malic acid and bacterial viable cell counts. Results show that there were significant differences between the amount of SO_2, acetaldehyde, and pyruvic acid produced by the various yeast strains but not α-ketoglutaric acid. Some yeast strains, such as FX10, S102, F15, and M69, produced significantly higher SO_2 concentrations than other yeast strains and *O. oeni* viability decreased rapidly when inoculated into these wines. Very little if any free SO_2 was measured indicating that bound SO_2 and not free was responsible for inhibition caused by SO_2. Acetaldehyde bound SO_2 was the dominant species of bound SO_2 found at almost all time points of the alcoholic fermentation, giving suggestive evidence that MLF inhibition was due to acetaldehyde bound SO_2.

INTRODUCTION

Malolactic fermentation (MLF) is a secondary fermentation that is a common practice in winemaking, particularly during red winemaking (Wibowo *et al.*, 1985; Lafon-Lafourcade *et al.*, 1983). It is preferably induced after alcoholic fermentation but may occur during the alcoholic fermentation (Versari *et al.*, 1999; Markides, 1993). It is the enzymatic decarboxylation of L-malic acid to L(+)-lactic acid and carbon dioxide (CO_2) by specific lactic acid bacteria (LAB) (Van Vuurren and Dicks, 1993; Wibowo *et al.*, 1985; Fleet *et al.*, 1984; Lafon-Lafourcade *et al*., 1983; Kunkee, 1967; Kunkee *et al.,* 1965). The most common LAB species used to conduct MLF is *Oenococcus oeni* (Versari *et al.*, 1999; Dicks *et al*., 1995; Davis *et al*., 1985; Wibowo *et al*., 1985) as this bacterium is well suited for growth in wines of low pH (< 3.5) (Versari *et al.*, 1999; Dicks *et al.*, 1995; Davis *et al.*, 1985; Wibowo *et al.*, 1985; Garvie, 1967).

Traditionally the practice relied on LAB naturally present on grapes or winery equipment to induce MLF, but the harsh environment of wine creates a very stressful growth medium for the bacteria and the MLF can be difficult to conduct. This problem was overcome to some extent by the development of select bacterial strains that were adapted to the low pH and high ethanol environment of wine (Alexandre *et al.*, 2004; Van Vuuren and Dicks, 1993; Davis *et al.*, 1985; Wibowo *et al.*, 1985). However, induction of the MLF can still be problematic due to a number of factors present during winemaking. Problematic MLFs are often attributed to the effects of low pH (Wibowo *et al*., 1988; Davis *et al*., 1985), temperature (Britz and Tracey, 1990), and/or antagonistic interactions between wine yeast (*Saccharomyces*) and malolactic bacteria (*Oenococcus*) (Henick-Kling and Park, 1994; Wibowo *et al*., 1988; Lonvaud-Funel *et al*., 1988; King

and Beelman, 1986; Beelman *et al*., 1982). Some researchers have proposed that inhibition of MLF is due to the removal of nutrients by the faster growing *Saccharomyces* (Beelman *et al*., 1982; Kunkee, 1967), nutrients that are required for the nutritionally fastidious malolactic bacteria (Garvie, 1967). However, recent studies have demonstrated that the removal of nutrients by yeast does not always explain the observed inhibition of *O. oeni* (Larsen *et al*., 2003; Patynowski *et al*., 2002).

Instead, other researchers have suggested that yeast may produce metabolites toxic to the malolactic bacteria (Osborne and Edwards, 2006; Capucho and San Ramao, 1994; Dick *et al.* 1992). These include ethanol (Davis *et al*., 1985; Costello *et al*., 1983), medium chain fatty acids (Versari *et al*., 1999; Edwards and Beelman 1987), antibacterial proteins/peptides (Osborne and Edwards, 2007; Dick *et al*., 1992), and yeast produced SO_2 (Larsen *et al*., 2003; Henick-Kling and Park, 1994; Eglinton and Henschke, 1996, King and Beelman, 1986). Of these compounds, SO_2 is most commonly implicated in causing bacterial inhibition (Larsen *et al*., 2003; Henick-Kling and Park, 1994; Fornachon, 1963) as it is a known antimicrobial against malolactic bacteria (Ough and Crowell, 1987; Amerine *et al*., 1980; Carr *et al*., 1976).

The production of SO_2 by yeast, coupled with that added to a must/wine, has been suggested by many researchers to be the primary mechanism of bacterial inhibition during alcoholic fermentation (Larsen *et al*., 2003; Henick-Kling and Park, 1994; Henick-Kling, 1993; Lonvaud-Funel *et al*., 1988; Fornachon, 1968). SO_2 is produced by *Saccharomyces* as an intermediate during the assimilatory reduction of sulfate to sulfite (Donalies and Stahl, 2002; Thomas and Surdin-Kerjan, 1997; Henick-Kling and Park,

1994). Depending on the needs and condition of the cell, yeast actively excrete sulfide via a membrane bound sulfite pump (Avram and Bakalinsky, 1997).

Once in the wine environment, SO_2 exists in equilibrium between molecular SO_2, bisulfite ions, and sulfite anions in a pH dependant manner with the dominant species at wine pH (3 to 4) being the bisulfite ion. Besides being in equilibrium with the molecular and sulfite species, bisulfite also exists in either a free or bound form (Fugelsang and Edwards, 2007). Here, the molecule will react with carbonyl compounds, such as acetaldehyde, forming addition products or adducts such as hydroxysulfonic acids. Although acetaldehyde binds most strongly with SO_2, other carbonyl compounds found in wine, such as pyruvic acid and α-ketoglutaric acid, may also bind with SO_2 (Azevedo *et al.,* 2007; Burroughs and Sparks, 1973; Rankine and Pocock, 1969; Fornachon, 1963).

Free SO_2 (and in particular the molecular portion) is considered to be the most antimicrobial among all the different forms of SO_2 (Rose and Pilkington, 1989; Hinze and Holzer, 1986; Eschenbruch, 1974; Hammond and Carr, 1976) with bound SO_2 thought to have much weaker antimicrobial properties. However, a number of researchers have suggested that bound SO_2 may be more antimicrobial than previously believed (Larsen *et al*., 2003; Henick-Kling and Park 1994; Hood, 1983; Fornachon, 1963). For example, Fornachon (1963) found that MLF was prevented in the presence of bound SO_2 where no free SO_2 was measurable. More recently, Larsen *et al*. (2003) and Osborne and Edwards (2006) found a strong correlation between yeast production of SO_2 and the inhibition of *O. oeni* in both synthetic media and in grape juice. These authors reported that all SO_2 measured was present in unidentified bound forms as free SO_2 was not

detected either by titration or capillary electrophoresis analysis. These findings suggest that bound forms of SO_2 could be much more antibacterial than previously thought.

The three major SO_2 binding compounds in wine, acetaldehyde, pyruvic acid, and α-ketoglutaric acid, are all produced by yeast during the alcoholic fermentation. Wine yeast have been shown to produce different amounts of acetaldehyde (Fleet and Heard, 1993), α-ketoglutarate (Rankine, 1968), and pyruvic acid (Rankine, 1965). Production of these SO_2 binding compounds is also dependent on pH (Rankine, 1967), grape variety (Rankine and Pocock, 1969), nitrogen source and thiamine deficiency (Rankine, 1965), and SO_2 concentration (Larsen *et al.*, 2003; Zoecklein *et al*., 1995; Williams, 1974; Burroughs and Sparks, 1973; Rankine and Pocock, 1969; Rankine, 1968; Lafon-Lafoucade and Peynaud, 1966; Fornachon, 1963). This will mean that depending what yeast strain conducts the alcoholic fermentation, there will be different amounts of the various SO_2 binding compounds present in the wine. Consequently there will be different concentrations of acetaldehyde bound SO_2, pyruvic acid bound SO_2, and α-ketoglutarate bound SO_2. However, the effect of the production of these compounds on the forms of bound SO_2 present in wine and the toxicity of these various forms to malolactic bacteria has not been investigated. If some forms of bound SO_2 are more inhibitory to malolactic bacteria than others, then this may explain the varied ability of wine yeast to inhibit the MLF despite the production of similar amounts of SO_2 (Larsen *et al.,* 2003; Osborne and Edwards, 2006). It may also explain why bacterial inhibition is strongest early in the alcoholic fermentation but decreases near the end or after completion of the fermentation (Larsen *et al.,* 2003).

Improved success in the induction of MLF requires a better understanding of the compatibility between different strains of yeast and malolactic bacteria. If different yeast strains produce different amounts of SO_2 binding compounds this may have to be accounted for when considering yeast/bacterial combinations. In addition the role bound SO_2 plays in causing problematic MLFs may need to be considered. Knowledge regarding the production of SO_2 binding compounds by yeast may also minimize SO_2 use through the choice of yeast strain based on the production of SO_2 binding compounds. For example, use of yeast strains that produce low amounts of binding compounds may allow the winemaker to add less SO_2 to maintain an effective level for antimicrobial purposes. This reduction of SO_2 use is beneficial, as there is mounting consumer resistance to the excessive use of SO_2 and other chemical preservatives in wine (Du Toit and Pretorious, 2000). Therefore the objective of this study was to investigate the ability of yeast strains to produce SO_2 and SO_2 binding compounds and the impact of the production of these compounds on the MLF at various time points during the alcoholic fermentation.

MATERIALS AND METHODS

Microorganisms

Active-dry forms of *Saccharomyces cerevisiae* obtained were strains MERIT.ferm and RUBY.ferm (Chr. Hansen, Hørsholm, Denmark), S102 and S325 (Laffort, France), V1116, M69, FX10, S6U, F15, BM45, and 43 (Lallemand, Montréal, Canada) and *S. bayanus* EC1118 (Lallemand). Yeast were maintained on potato dextrose agar (PDA) (Difco, Franklin Lakes, NJ, USA) slants stored at 4°C. The strain *Oenococcus oeni* used in this study was the freeze-dried form of DSM 7008, Viniflora oenos (Chr. Hansen).

Starter culture preparation

Yeast were transferred from PDA slants to 250 mL yeast peptone dextrose (YPD) broth (10g/L yeast extract, 20g/L peptone, 20g/L dextrose, pH 7.0) and incubated aerobically at 25°C for 48h. Cells were harvested by centrifugation (4,000 x *g* for 20 minutes) and resuspended in 0.2 M phosphate buffer (27.80 g/L $NaH_2PO_4 \cdot H_2O$, 28.38 g/L Na_2HPO_4, pH 7.0) before inoculation at a rate of $1x10^6$ CFU/mL. To initiate malolactic fermentation, freeze-dried bacteria were rehydrated with 0.2 M phosphate buffer and directly inoculated at a rate of $1x10^6$ CFU/mL.

Enumeration

Microbial viabilities were determined using diluents containing 0.1% peptone and using appropriate media. Yeast cells were grown on YPD agar while bacteria were enumerated using de Man, Rogosa, and Sharpe (MRS) agar (20g/L Tryptone, 5g/L peptone, 5g/L yeast extract, 5g/L glucose, 200mL apple juice, 1mL Tween 80 [5% w/w solution], 20 g/L agar, pH 4.5). Plates were incubated aerobically at 25°C for 48h (yeast) or 7 days (bacteria) prior to counting.

Synthetic Grape Juice

A synthetic grape juice based on Wang *et al*. (2003) and modified as per Osborne and Edwards (2006) was utilized (Appendix A), with a concentration of 250 mg/L yeast assimilable nitrogen (YAN). YAN was calculated as the sum of the concentrations of ammonia and the molar proportion of the α-amino nitrogen present in amino acids except proline.

Pinot Gris Juice

Pinot gris grapes harvested from Oregon State University's Woodhall Vineyard (2008) were pressed and the juice was stored at -20°C until needed. No SO_2 was added to the juice following pressing.

Alcoholic fermentation

Both the synthetic grape juice and Pinot gris grape juice were sterile filtered using 0.45µm PES disposable Ultripor® filters (PALL Corp. East Hills, NY, USA) into sterile 1 gallon glass carboys. The final volume for each fermentor was 3L. Alcoholic fermentations were induced by the addition of yeast at a rate of $1x10^6$ CFU/mL. All fermentations were performed in triplicate and fermentations were performed at 21°C. Aseptic sampling during the fermentation was accomplished using a nitrogen siphon system. Completion of the alcoholic fermentation was determined by CliniTest®.

Fermentation monitoring

During alcoholic fermentation, 150 mL samples were aseptically removed and 100 mL was sterile filtered through 0.45µm disposable Nalgene PES membrane filter unit (NalgeNuno International, Rochester, NY, USA) into sterilized milk dilution bottles. Freeze-dried *O. oeni* VFO were rehydrated in 0.2 M phosphate buffer for 10 minutes before inoculation into the 100 mL sterile filtered samples at initial populations of approximately $1x10^6$ CFU/mL. Bottles were incubated at 25°C with bacterial viable cell populations and L-malic acid (enzymatic test kit, R-Biopharm, Darmstadt, Germany) being measured weekly.

The remaining samples (≈50 mL) were used to analyze yeast viable cell populations and free/total SO_2 by the aeration-oxidation method (Buechsenstein and Ough, 1978). Samples were also analyzed for acetaldehyde and pyruvic acid (enzymatic test kit, R-Biopharm) and α-ketoglutarate (enzymatic assay as described by Peynaud *et al*. (1966)).

Calculating metabolite-bound SO_2

Upon completion of the alcoholic fermentation the concentration of metabolite-bound SO_2 was determined using an equation formulated by Burroughs and Sparks (1964), as shown below, to determine the amount of each binding compound that would be bound to SO_2.

$$[x] = [X] \times \frac{[S]}{K + [S]}$$

Where: [S] = molecular concentration of free SO_2 in any form;
[X] = *total* molecular concentration of the carbonyl compound (free + bound)
[x] = molecular concentration of undissociated carbonyl bisulfite
K = equilibrium constant for specific binding compound

Statistics

Statistical analysis was accomplished using SAS version 9.1 (SAS Institute Inc, Cary, NC, USA) with Tukey's HSD test for mean separation.

RESULTS

After inoculation into the synthetic grape juice all yeast strains achieved populations of $1x10^8$ CFU/mL or greater within 10 days (Figures 3.1). Exponential growth occurred within 2 days after inoculation with yeast strains BM45 and FX10 reaching the highest cell populations of almost $1x10^9$ CFU/mL (Figure 3.1B). Stationary cell growth was achieved within day 3 and maintained until day 15, after which the viable cell count for all yeast declined slowly until reaching a minimum of around $1x10^5$ CFU/mL by day 50.

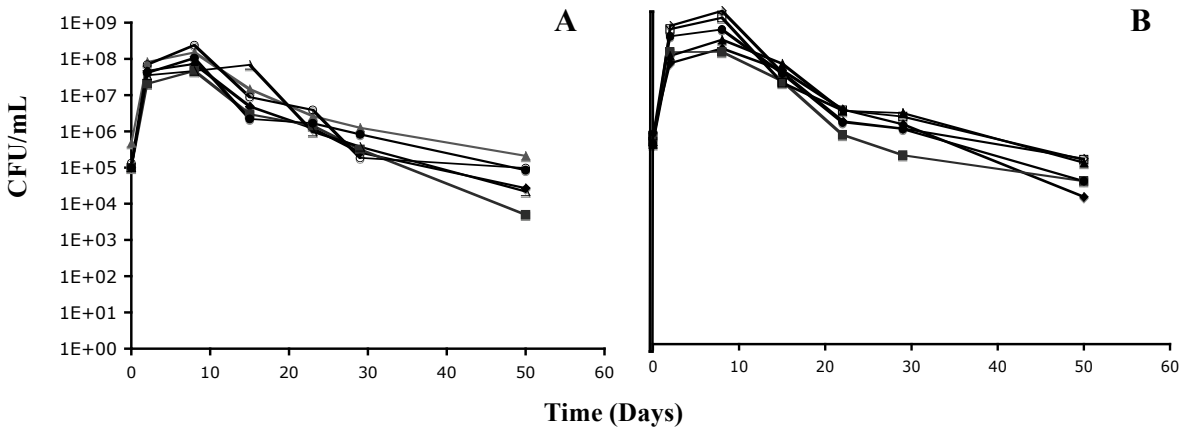


Figure 3.1: Growth of *S. cerevisiae* strain (**A**) V1116 ◆; RUBY.ferm ■; MERIT.ferm ▲; S325 ●; EC1118 ○; S102 △ (**B**) M69 ◆; FX10 ■; S6U ▲; 43 ●; F15 ◇; BM45 □ during the alcoholic fermentation in a synthetic grape juice. Values are means of triplicate fermentations.

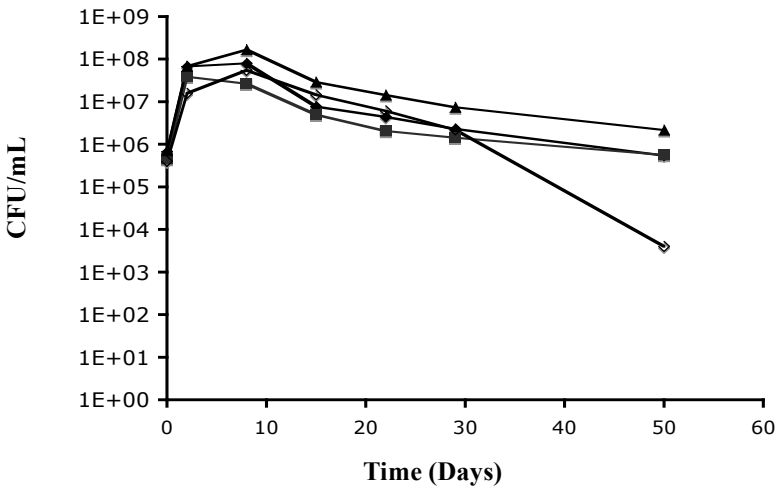


Figure 3.2: Growth of *S. cerevisiae* strain V1116 ◆; FX10 ■; BM45 ▲; M69 ◇ during the alcoholic fermentation in a Pinot gris juice. Values are means of triplicate fermentations

Results from the Pinot gris juice are similar to the synthetic medium in terms of exponential and stationary growth (Figure 3.2). Exponential growth occurred within 2 days of fermentation and achieved stationary phase by day 3. As in the synthetic media, yeast BM45 reached the highest cell population of $1x10^8$ CFU/mL by day 10 of fermentation. Interestingly strain M69 decreased in viable cell counts between sample day 29 and 50 by 100-fold, from $1x10^6$ CFU/mL to $1x10^4$ CFU/mL whereas the other three strains did not show such dramatic decline in viability.

Despite no observable differences in yeast growth, with the exception of strain M69 in the Pinot gris juice, yeast strains produced varying amounts of total SO_2. Overall, the concentrations of free SO_2 in the synthetic grape juice were minimal, ranging from 2.13 mg/L by strain MERIT.ferm to 9.07 mg/L by strain BM45 15 days after inoculation (Table 3.1A, Figure 3.3).

Results from the Pinot gris juice (Figure 3.4) are similar to what was observed in the synthetic grape juice where very small concentrations of free SO_2 were measured

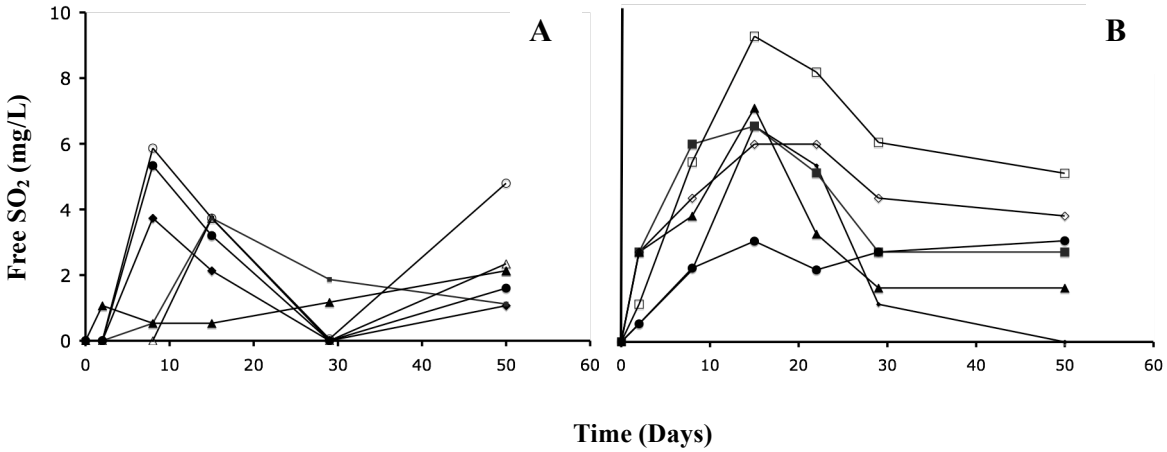


Figure 3.3: Concentrations of free SO_2 during alcoholic fermentation of a synthetic grape juice by *S. cerevisiae* (**A**) V1116 ◆; RUBY.ferm ■; MERIT.ferm ▲; S325 ●; EC1118 ○; S102 △ (**B**) M69 ◆; FX10 ■; S6U ▲; 43 ●; F15 ◇; BM45 □. Values are means of triplicate fermentations.

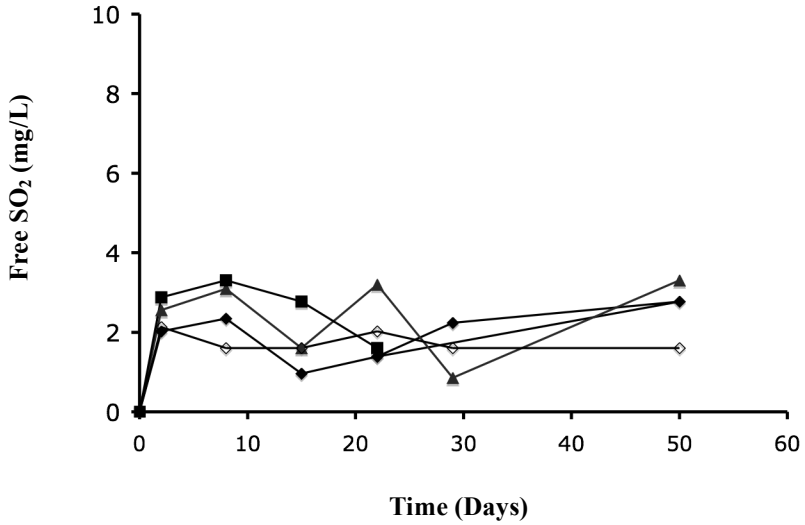


Figure 3.4: Concentrations of free SO_2 during alcoholic fermentation of Pinot gris by *S. cerevisiae* V1116 ◆; FX10 ■; BM45 ▲; M69 ◇. Values are means of triplicate fermentations.

during the alcoholic fermentation. Both FX10 and BM45 produced a maximum of 3.31 mg/L free SO_2 (Table 3.1B, Figure 3.4). Overall, lower amounts of free SO_2 were observed during the Pinot gris fermentation compared to the synthetic media when the same yeast strain was used to conduct the fermentations.

Table 3.1: Maximum concentrations of free and bound SO_2, acetaldehyde, pyruvic acid, and α-ketoglutarate measured during fermentation by *S. cerevisiae* yeast strains in a synthetic medium (A) and Pinot gris juice (B) [1] (mg/L).

A	Free SO_2	Bound SO_2	Acetaldehyde	Pyruvic Acid	α-ketoglutarate	MLF Inhibition
V1116	2.7^{b}	31.5^{c}	$102.4^{a\,b}$	90.0^{b}	39.2^{a}	- - -
RUBY.ferm	3.7^{b}	27.7^{c}	$47.9^{c\,d}$	103.6^{b}	32.2^{a}	- -
MERIT.ferm	2.1^{b}	28.8^{c}	$71.9^{b\,c\,d}$	$113.7^{a\,b}$	37.2^{a}	+
EC1118	$5.9^{a\,b}$	28.8^{c}	$89.5^{a\,b}$	98.4^{b}	30.7^{a}	- -
S102	3.7^{b}	$36.3^{a\,b}$	$89.8^{a\,b}$	$113.7^{a\,b}$	41.0^{a}	- - -
S325	$5.3^{a\,b}$	32.0^{c}	$74.5^{a\,b\,c}$	$115.3^{a\,b}$	45.8^{a}	- -
M69	$5.2^{a\,b}$	60.3^{a}	$44.4^{c\,d}$	$120.0^{a\,b}$	37.7^{a}	- -
FX10	$5.9^{a\,b}$	50.7^{a}	35.0^{d}	$113.8^{a\,b}$	39.0^{a}	- - -
S6U	$6.9^{a\,b}$	$38.9^{a\,b\,c}$	$37.1^{c\,d}$	$112.5^{a\,b}$	43.6^{a}	+
F15	$5.9^{a\,b}$	$49.4^{a\,b}$	$44.2^{c\,d}$	$175.8^{a\,b}$	47.1^{a}	- - -
BM45	9.0^{a}	$39.5^{a\,b\,c}$	$49.8^{c\,d}$	$136.1^{a\,b}$	42.9^{a}	- - -
43	3.5^{b}	$41.1^{a\,b\,c}$	109.7^{a}	223.5^{a}	31.5^{a}	+
B						
V1116	2.8^{a}	43.8^{a}	75.0^{a}	86.9^{a}	62.6^{a}	- - -
FX10	3.3^{a}	47.7^{a}	74.5^{a}	47.0^{a}	48.8^{a}	- -
BM45	3.3^{a}	42.6^{a}	89.8^{a}	86.7^{a}	57.2^{a}	- - -
M69	2.1^{a}	33.1^{a}	105.1^{a}	68.6^{a}	45.7^{a}	+

[1] Values are means of triplicate fermentations

$^{a-d}$ Values with no common superscripts are significantly different (Tukey's Studentized Range Test $p<0.05$)

- - - No degradation of malic acid: MLF ≥ 28 days

- - Delayed degradation of malic acid: MLF completed between 14 and 28 days

\+ Rapid degradation of malic acid: MLF complete ≤ 14 days

Although low free SO_2 concentrations were observed during the fermentations, much higher levels of bound SO_2 were noted during alcoholic fermentation in both the synthetic grape juice (Figure 3.5) as well as the Pinot gris juice (Figure 3.6).

For the synthetic grape juice, maximum concentrations of bound SO_2 ranged from 27.73 mg/L in fermentations induced by RUBY.ferm to 60.26 mg/L in fermentations induced by M69 (Table 3.1). Maximum production of SO_2 occurred by day 8 of the fermentation (Figure 3.5). A reduction in the amount of bound SO_2 occurred after this peak for most yeast strains although fermentations conducted by strains V1116, RUBY.ferm, S325, EC1118, and S102 displayed little reduction of bound SO_2. Finally, an increase in bound SO_2 was observed from day 29 to day 50 for most strains except MERIT.ferm (Figure 3.5A), in which there was no increase.

Results from fermentations in Pinot gris juice produced similar results to those observed in the synthetic grape juice (Figure 3.6). Maximum concentrations of bound SO_2 measured during the fermentation ranged from 33.07 mg/L (M69) to 47.68 mg/L (FX10) as shown in Table 3.1A. Compared to fermentation in synthetic grape juice M69 fermentations in Pinot gris juice contained lower concentrations of bound SO_2.

In addition to SO_2, the major SO_2 binding compounds were also measured during the course of the fermentations. The production of acetaldehyde by *S. cerevisiae* is shown in Figure 3.7. Results display that each yeast strain produced different concentrations of acetaldehyde throughout the alcoholic fermentation, with production peaking at the beginning of fermentation (day 2) during yeast exponential growth phase. In general, the concentration of acetaldehyde decreased as the fermentation proceeded.

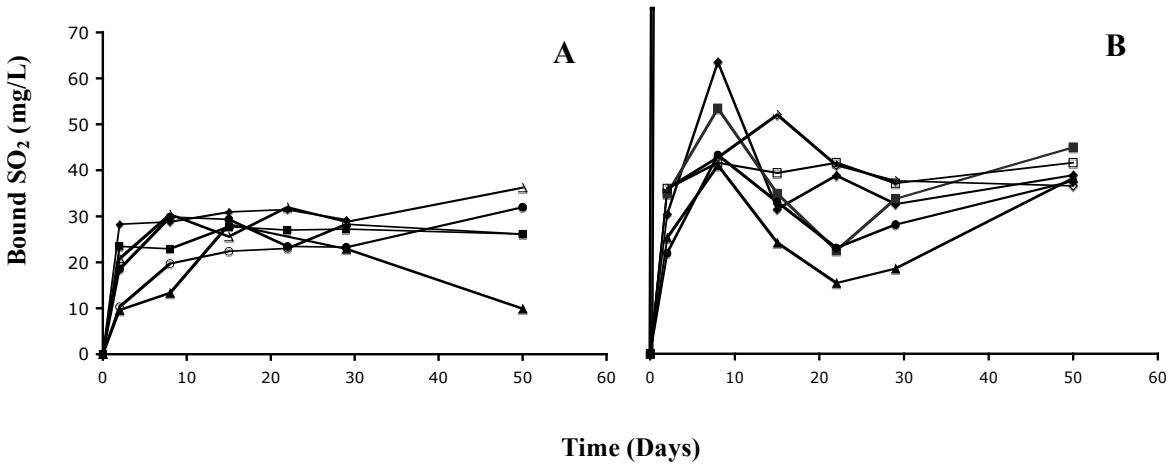


Figure 3.5: Concentrations of bound SO_2 during alcoholic fermentation of a synthetic grape juice induced by *S. cerevisiae* strain (**A**) V1116 ◆; RUBY.ferm ■; MERIT.ferm ▲; S325 ●; EC1118 ○; S102 △ (**B**) M69 ◆; FX10 ■; S6U ▲; 43 ●; F15 ◇; BM45 □. Values are means of triplicate fermentations.

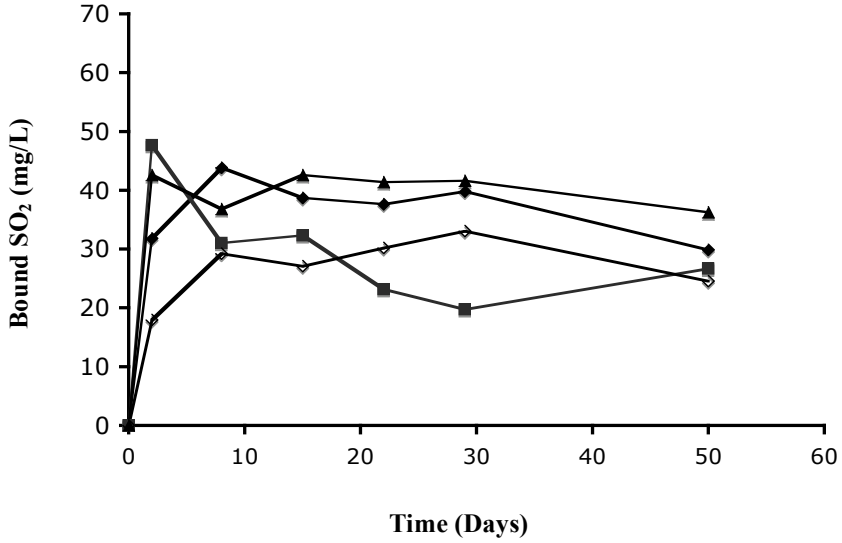


Figure 3.6: Concentrations of bound SO_2 during alcoholic fermentation of Pinot gris by *S. cerevisiae* V1116 ◆; FX10 ■; BM45 ▲; M69 ◇. Values are means of triplicate fermentations.

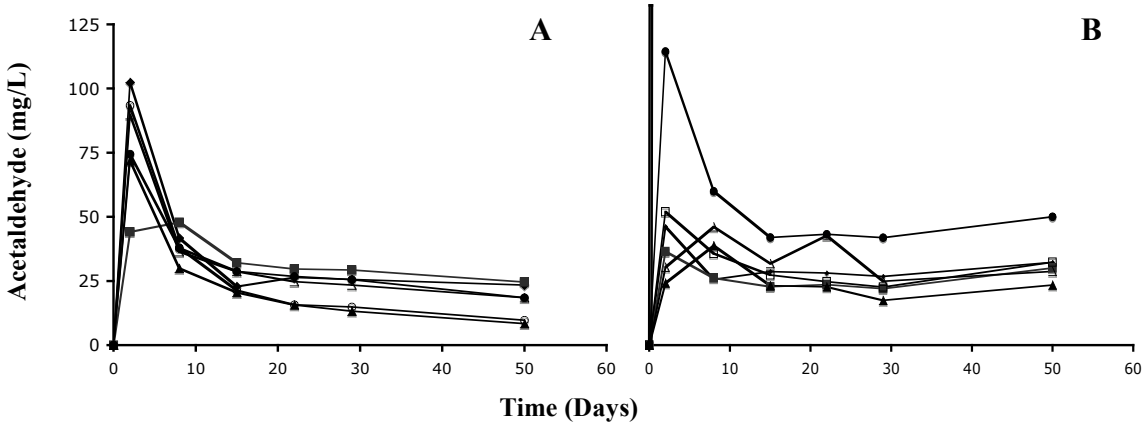


Figure 3.7: Concentration of acetaldehyde throughout the alcoholic fermentation in a synthetic juice induced by *S. cerevisiae* strain (**A**) V1116 ◆; RUBY.ferm ■; MERIT.ferm ▲; S325 ●; EC1118 ○; S102 △ (**B**) M69 ◆; FX10 ■; S6U ▲; 43 ●; F15 ◇; BM45 □. Values are means of triplicate fermentations.

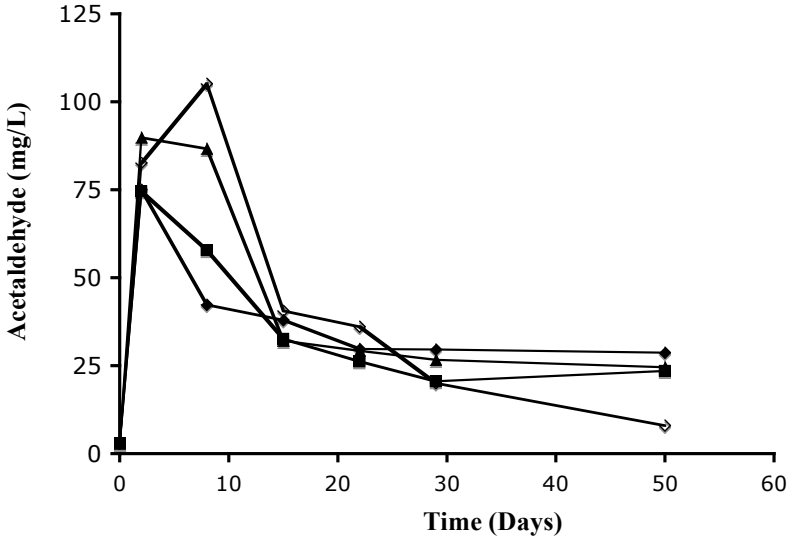


Figure 3.8: Concentration of acetaldehyde throughout the alcoholic fermentation in a Pinot gris juice induced by V1116 ◆; FX10 ■; BM45 ▲; M69 ◇. Values are means of triplicate fermentations.

Yeast strain 43 produced the highest amount of acetaldehyde (109.67 mg/L) followed by V1116 (102.42 mg/L), while strain FX10 produced the lowest concentration of acetaldehyde (35 mg/L) as displayed in Table 3.1A.

Figure 3.8 shows the production of acetaldehyde by yeast strains in a Pinot gris juice. Results are similar to those observed in the synthetic grape juice where large concentrations of acetaldehyde were produced at the beginning of the alcoholic fermentation with strain M69 producing a maximum of 105.08 mg/L (Table 3.1B) by day 8. It is interesting to note that at the end of the alcoholic fermentation acetaldehyde levels in wine produced by strain M69 was the lowest (Figure 3.8). The other strains produced similar total acetaldehyde concentrations at each time point of the alcoholic fermentation, with the exception of day 8 (Figure 3.8). Except for V1116, yeast strains produced higher amounts of acetaldehyde during fermentation in Pinot gris juice then they had during fermentation in synthetic media (Table 3.1).

Pyruvic acid was also measured during the course of the fermentations and the results are exhibited in Figure 3.9 and Figure 3.10 for both the synthetic grape juice and Pinot gris juice fermentations, respectfully. In the synthetic grape juice, trends in pyruvic acid production appear to be similar to acetaldehyde production where the maximum amount of pyruvic acid present occurred early in the fermentation during active growth by the yeast. However, unlike acetaldehyde concentrations the concentration of pyruvic acid did not decline as the fermentation continued. One exception to this trend was yeast strain F15. During fermentation in synthetic grape

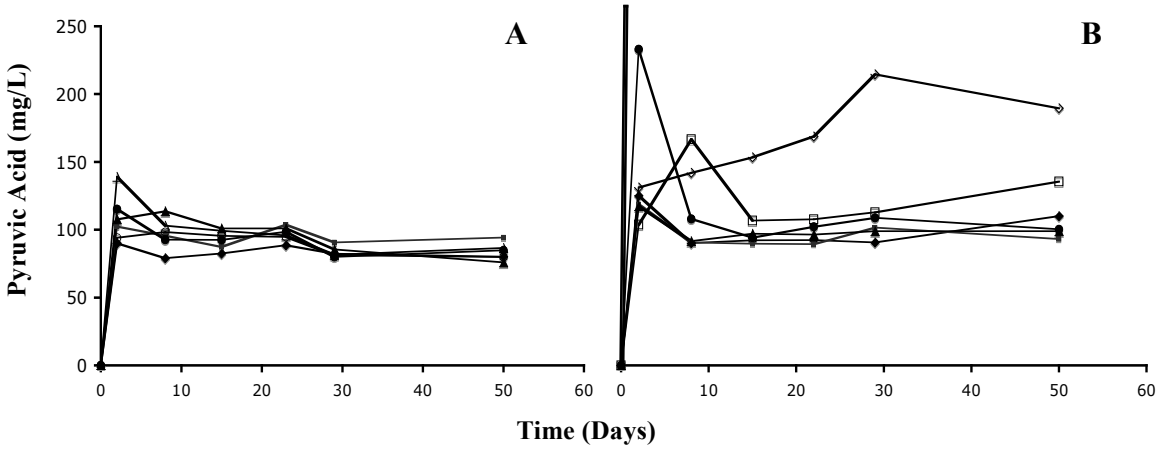


Figure 3.9: Concentration of pyruvic acid throughout the alcoholic fermentation in a synthetic juice induced by (**A**) ◆ V1116; ■ RUBY.ferm; ▲ MERIT.ferm; ● S325; ○ EC1118; △ S102 (**B**) ◆ M69; ■ FX10; ▲ S6U; ● 43; ◇ F15; □ BM45. Values are means of triplicate fermentations.

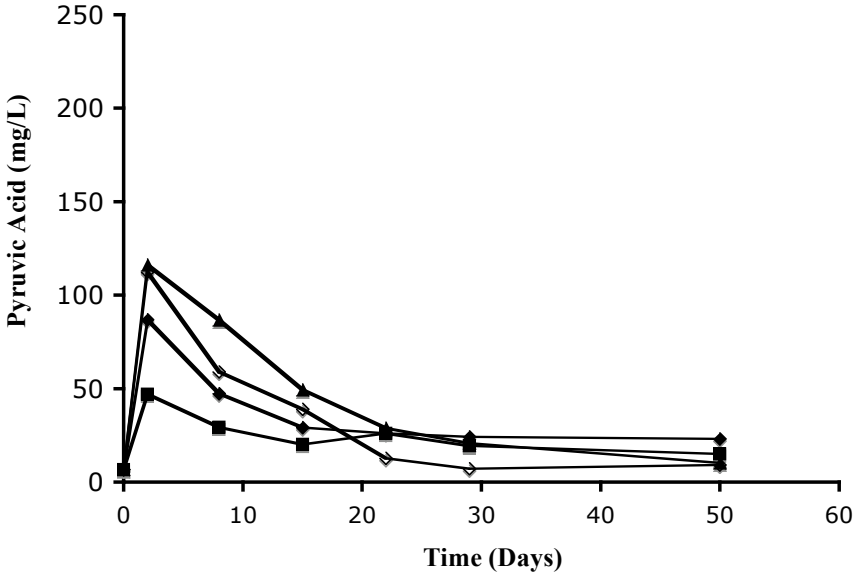


Figure 3.10: Concentration of pyruvic acid formed the alcoholic fermentation in a Pinot gris juice induced by V1116 ◆; FX10 ■; BM45 ▲; M69 ◇. Values are means of triplicate fermentations.

juice this yeast strain produced a maximum concentration of pyruvic acid (175.79 mg/L) much later in the fermentation (day 28) compared to the other strains (Figure 3.9B). Yeast strain 43 produced the most pyruvic acid during fermentation in synthetic grape juice as seen in Figure 3.9B and Table 3.1A with a maximum production of 223.51 mg/L pyruvic acid 2 days after inoculation.

Results from the Pinot gris must are similar to the synthetic grape juice fermentations in that the concentration of total pyruvic acid spiked within 2 days of fermentation. However, unlike the synthetic grape juice, the concentration of total pyruvic acid decreased as the alcoholic fermentation progressed with fermentations conducted by M69 reaching a minimum of 5.00 mg/L on day 29 (Figure 3.10). In contrast to what was observed for acetaldehyde all yeast strains, except V1116, produced lower concentrations of pyruvic acid during fermentation in Pinot gris juice then they had in synthetic grape juice (Table 3.1).

Concentrations of α-ketoglutarate produced by each yeast strain followed a similar pattern to that of acetaldehyde and pyruvic acid production (Figure 3.11, 3.12) although smaller differences between yeast strains were observed. In both the synthetic grape juice and the Pinot gris grape juice, the maximum α-ketoglutarate production did not exceed 70 mg/L (Figure 3.11, Figure 3.12, Table 3.1). In the synthetic grape juice strain F15 produced a maximum of 47.05 mg/L α-ketoglutarate whereas strain 43 produced the lowest concentration of 31.48 mg/L (Table 3.1A).

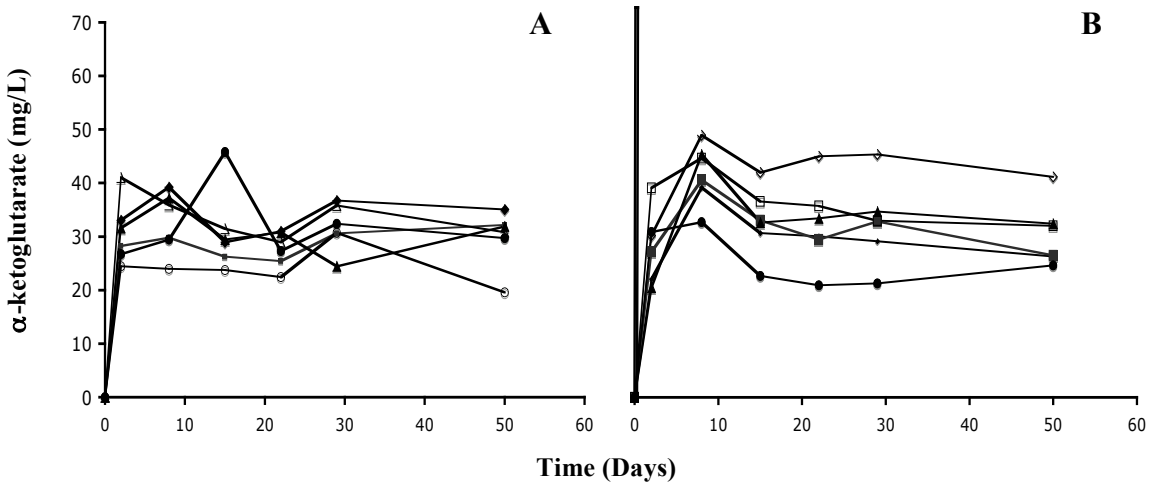


Figure 3.11: Concentration of α-ketoglutarate throughout the alcoholic fermentation in a synthetic juice induced by (**A**) V1116 ◆; RUBY.ferm ■; MERIT.ferm ▲; S325 ●; EC1118 ○; S102 △ (**B**) M69 ◆; FX10 ■; S6U ▲; 43 ●; F15 ◇; BM45 □. Values are means of triplicate fermentations.

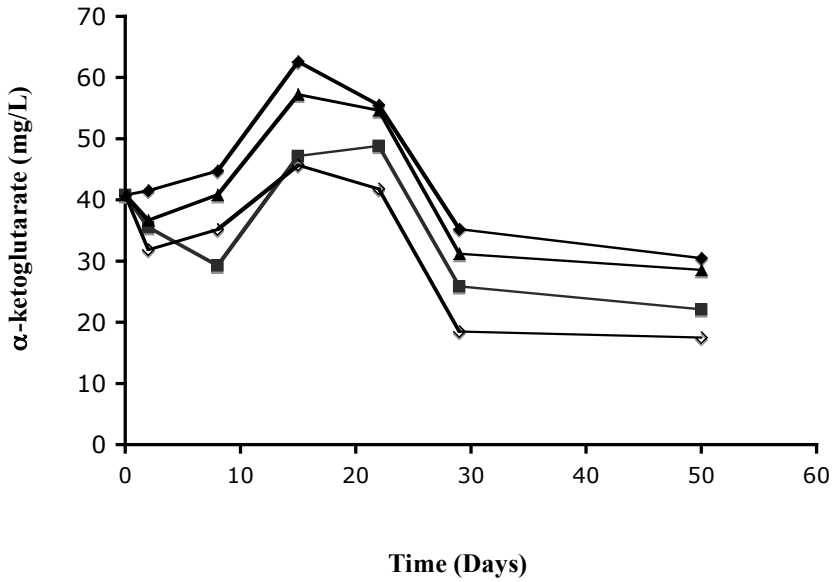


Figure 3.12: Concentration of α-ketoglutarate throughout the alcoholic fermentation in a Pinot gris juice induced by V1116 ◆; FX10 ■; BM45 ▲; M69 ◇. Values are means of triplicate fermentations.

In the Pinot gris juice, strain V1116 produced a maximum of 62.63 mg/L α-ketoglutarate (Table 3.1B) whereas strain M69 produced the lowest concentration of α-ketoglutarate (25 mg/L) (Figure 3.12). Overall, α-ketoglutarate concentrations were higher in the Pinot gris fermentations than in the synthetic grape juice fermentations.

The bacterium grew well in the synthetic grape juice after 0 days of alcoholic fermentation (Appendix C, D) and malic acid was consumed in samples fermented by MERIT.ferm and EC1118 (Figure 3.13) and S6U and 43 (Figure 3.14) at every subsequent sampling day. However, decreases in bacterial viability (Appendix C, D) and slower malic acid utilization were observed in samples fermented by V1116, S102, S325 (Figure 3.13), FX10, M69, BM45, and F15 (Figure 3.14) that were removed after 2, 8, 15, 23, 29, and 50 days.

In the Pinot gris juice, *O. oeni* VFO grew well (Appendix E) and malic acid was consumed in musts fermented by M69 and FX10 (Figure 3.15) but bacterial viability rapidly decreased (Appendix E) and slower malic acid utilization were observed in must/wine fermented by V1116 and FX10 (Figure 3.15) for 15, 22, 29, and 50 days.

A summary of whether or not MLF was inhibited is shown in Table 3.1. As can be seen in the table, MLF was not inhibited in synthetic grape juice fermented by MERIT.ferm, S6U, and 43 but was inhibited in synthetic grape juice fermented by V1116, S102, FX10, F15, and BM45. In Pinot gris fermentations, MLF was inhibited by V1116, FX10, and BM45 but was not inhibited in must/wine fermented by M69.

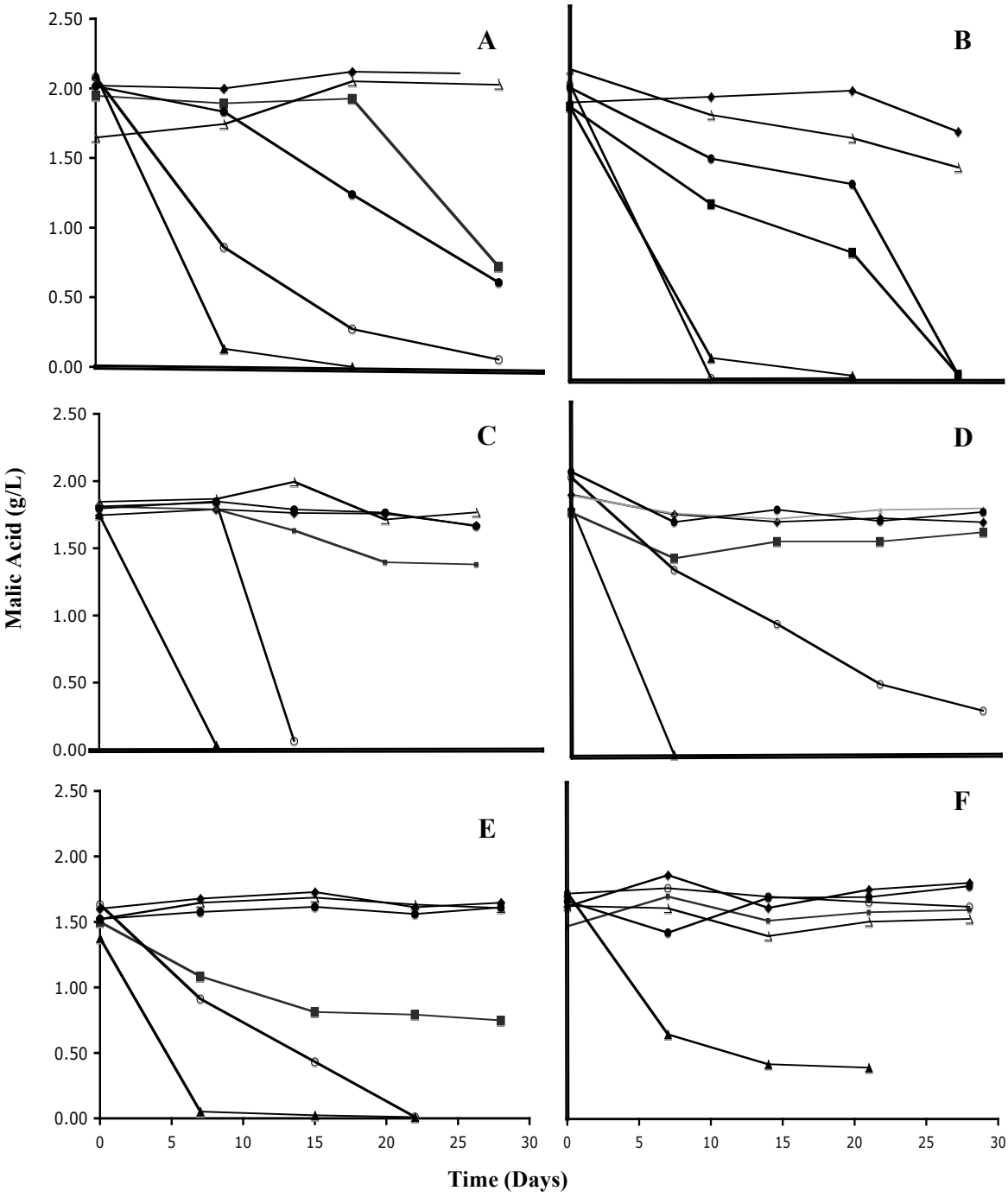


Figure 3.13: Malic acid degradation by *O. oeni VFO* in a synthetic medium undergoing alcoholic fermentation induced by yeast strains V1116 ◆; RUBY.ferm ■; MERIT.ferm ▲; S325 ●; EC1118 ○; S102 △. Samples from each yeast fermentation were removed on day 2 (A), 8 (B), 15 (C), 23 (D), 29 (E), and 50 (F) of alcoholic fermentation, sterile filtered, and inoculated with the bacterium. Values are means of triplicate fermentations.

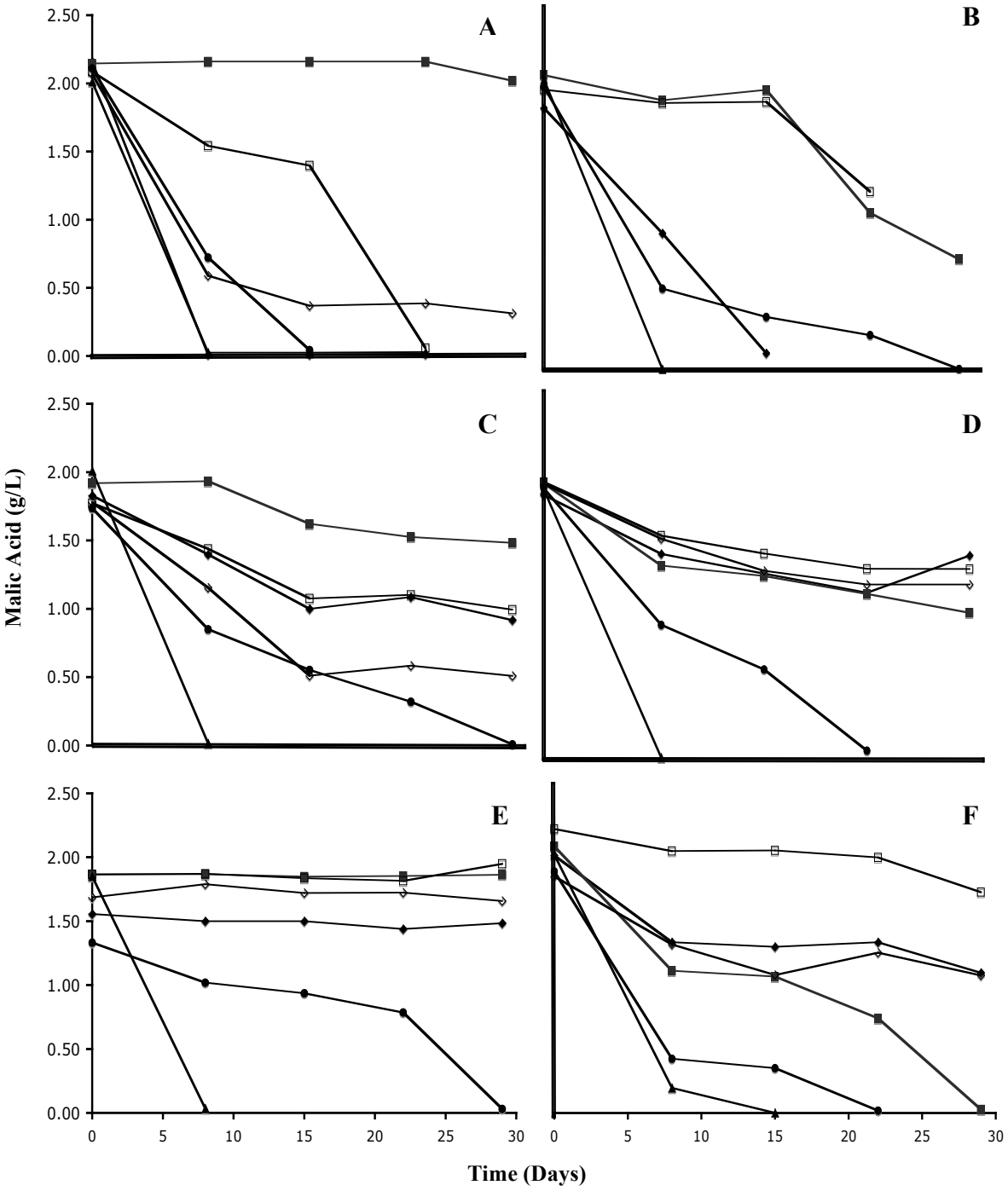


Figure 3.14: Malic acid degradation by *O. oeni VFO* in a synthetic medium undergoing alcoholic fermentation induced by strains M69 ◆; FX10 ■; S6U ▲; 43 ●; F15 ◇; BM45 □. Samples from each yeast fermentation were removed on day 2 (A), 8 (B), 15 (C), 23 (D), 29 (E), 50 (F) of alcoholic fermentation, sterile filtered, and inoculated with the bacterium. Values are means of triplicate fermentations.

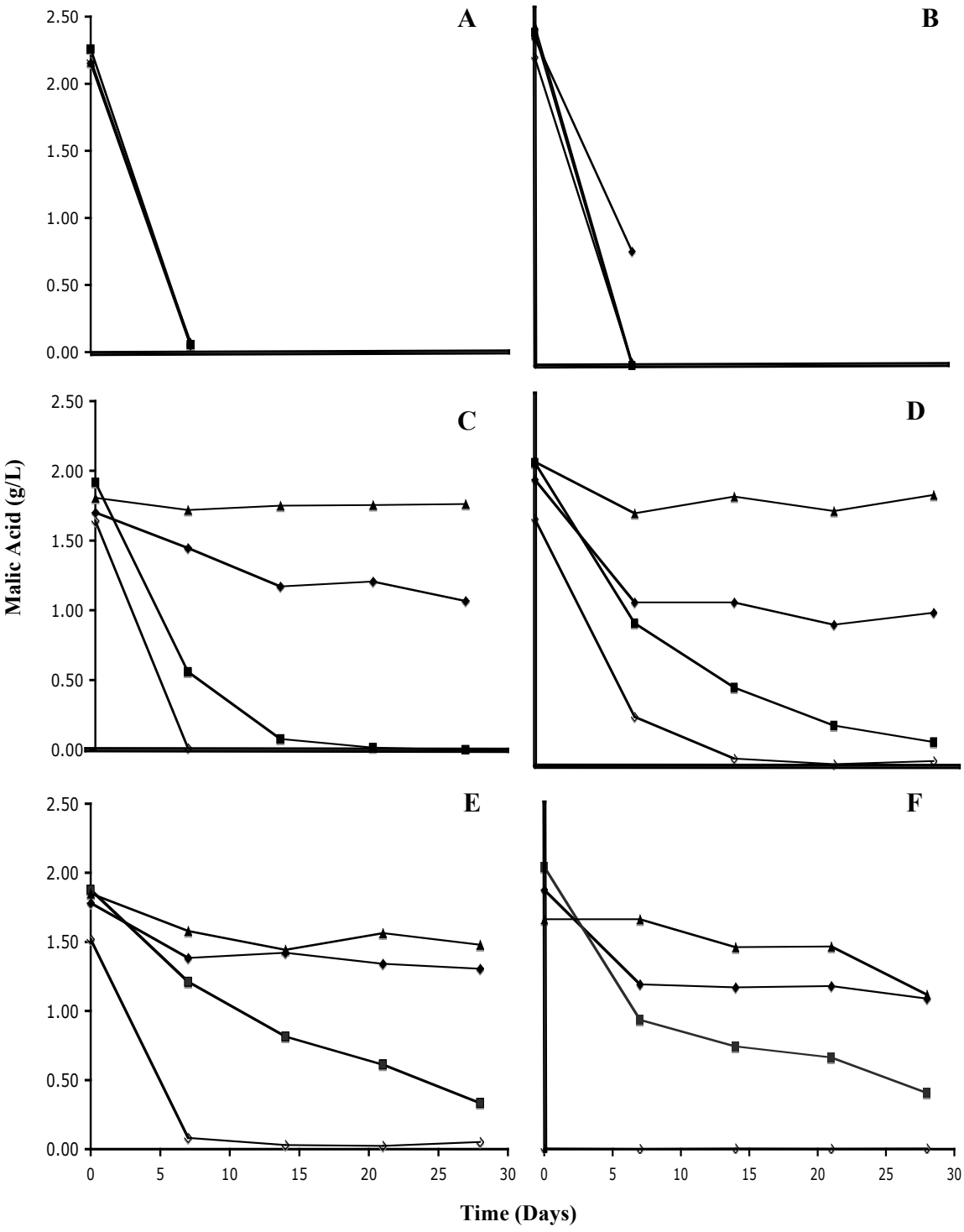


Figure 3.15: Malic acid degradation by *O. oeni VFO* in Pinot gris juice undergoing alcoholic fermentation induced by strains V1116 ◆; BM45 ▲; FX10 ■; M69 ◇. Samples from each yeast fermentation were removed on day 2 (A), 8 (B), 15 (C), 23 (D), 29 (E), and 50 (F) of alcoholic fermentation, sterile filtered, and inoculated with the bacterium. Values are means of triplicate fermentations.

The results (Table 3.2, 3.3, Figures 3.16, 3.17, Appendix E - JJ) show that at most points of the alcoholic fermentation, only acetaldehyde bound SO_2 was present. On a molar basis there were higher concentrations of acetaldehyde than SO_2 during alcoholic fermentation of the Pinot gris juice by almost all yeast strains. This would mean that the bound SO_2 would all have been present as acetaldehyde bound SO_2. For example in Figure 3.16, 100% of SO_2 is bound to acetaldehyde for *S. cerevisiae* strain FX10 during the fermentation of Pinot gris juice. This was also the case for most of the fermentations performed in synthetic media. However strain FX10 displayed four time points that had a higher concentrations of SO_2 than acetaldehyde (days 8, 15, 28, and 50) indicating that pyruvic acid bound SO_2 would have also been present at these times (Figure 3.17).

Table 3.2: Bound SO_2 and calculated binding (mg/L) compounds in Pinot gris juice during alcoholic fermentation for *S. cerevisiae* strain FX10.

Alcoholic Fermentation Day	Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	47.68	47.68	0.00	0.00
8	31.04	32.01	0.00	0.00
15	32.32	33.23	0.00	0.00
22	23.15	23.15	0.00	0.00
29	19.73	19.73	0.00	0.00
50	26.67	26.30	0.00	0.00

[1] Values are means of triplicate samples.

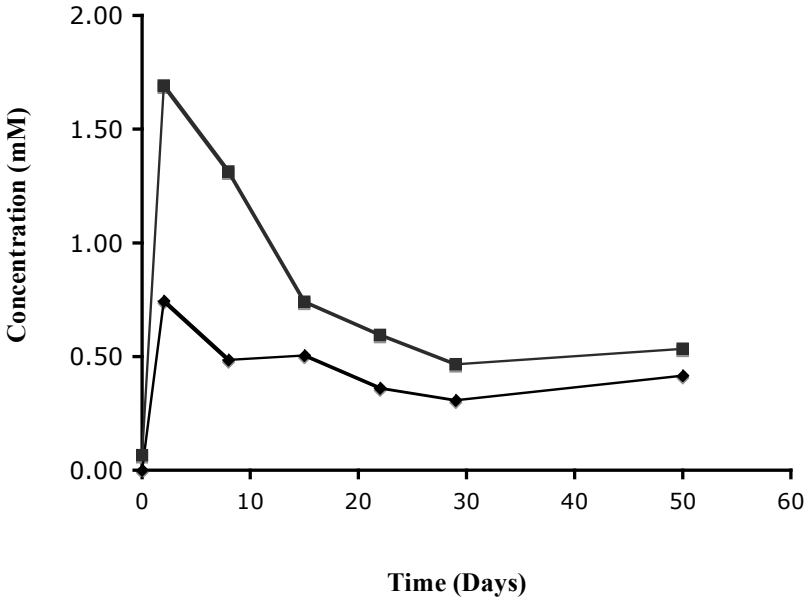


Figure 3.16: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain FX10 in Pinot gris juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

Table 3.3: Bound SO_2 and calculated binding (mg/L) compounds in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain FX10.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0	0.00	0.00
2	33.07	33.07	0.00	0.80
8	50.67	35.90	23.47	2.42
15	33.07	31.22	1.85	0.00
22	21.33	22.87	0.00	0.00
29	32.00	29.62	2.00	0.97
50	42.67	41.16	1.68	0.46

[1]Values are means of triplicate samples.

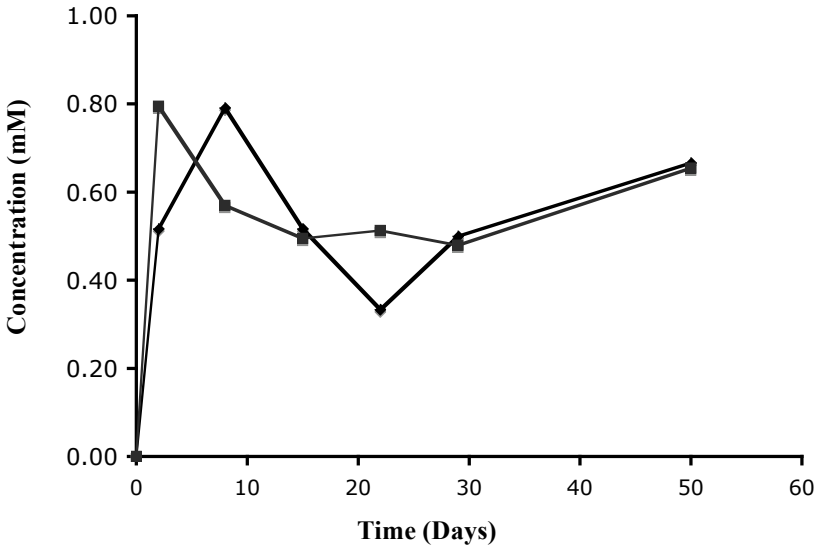


Figure 3.17: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain FX10 in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

DISCUSSION

During alcoholic fermentation in a synthetic grape juice and a Pinot gris grape juice, various strains of *S. cerevisiae* produced different amounts of SO_2. As reported by Rankine and Pocock, (1969), yeast can produce a wide range of SO_2 concentrations with the ability of *S. cerevisiae* wine yeast to produce SO_2 being dependent upon various factors, including the strain involved, fermentation temperature, and the wine's composition (Osborne and Edwards, 2006; Jarvis and Lea, 2000; Henick-Kling, 1994; Würdig, 1989; Eschenbruch, 1974; Weeks, 1969). In this present study, juice composition and fermentation temperatures were kept constant and so the differences in SO_2 production observed were therefore primarily due to yeast strain differences.

Aside from differences in the production of SO_2, yeast also differed in their production of the major SO_2 binding compounds. While differences in yeast production of acetaldehyde has previously been reported by many others (Larsen *et al*., 2003; Martinez *et al*., 1997; Ough, 1985; Margalith, 1981; Weeks, 1969; Ribereau-Gayon *et al,* 1956), the production of pyruvic acid and α-ketoglutarate by wine yeast during alcoholic fermentation is less well documented (Rankine, 1968; Rankine, 1967; Rankine, 1965). In addition, this present study documents the production of all three of the major SO_2 binding compounds as well as SO_2 at numerous times during the course of the alcoholic fermentation rather than final concentrations in the finished wines as was reported in previous studies (Rankine, 1968; Rankine, 1967; Rankine, 1965).

For acetaldehyde, maximum production occurred during yeast exponential growth phase in agreement with previous findings (Martinez *et al*., 1997; Margalith, 1981; Amerine and Ough, 1964; Weeks, 1969; Ribereau-Gayon *et al,* 1956). Although other

researchers have reported that the presence of SO_2 can induce the production of acetaldehyde (Henick-Kling *et al*., 1998; Pilkington and Rose, 1988; Stratford *et al*., 1987; Rankine and Pocock, 1969; Weeks, 1969), this was not always observed in the present study. For example, during fermentation in Pinot gris juice yeast strain M69 produced the lowest amount of SO_2 but the highest amount of acetaldehyde. The SO_2 induced production of acetaldehyde has been suggested to be a mechanism that yeast use to tolerate higher SO_2 concentrations. (Pilkington and Rose, 1988; Stratford *et al*., 1987). This response was not apparent in this study. Further research on this topic is needed to determine the link between SO_2 and the production of acetaldehyde by *S. cerevisiae*.

Differences in yeast production of pyruvic acid and α-ketoglutarate were minimal with yeast strain 43 the only strain to produce significantly higher concentrations of pyruvic acid. The concentrations produced were in agreement with the findings of Rankine (1967) and Rankine (1968). Maximum production of pyruvic acid and α-ketoglutarate generally occurred during yeast exponential growth phase as would be expected as these compounds are intrinsically involved in yeast metabolism. Interestingly, higher concentrations of acetaldehyde, pyruvic acid, and α-ketoglutarate were measured during fermentation in the synthetic grape must than the Pinot gris juice. This may have been due to differences in pH, nitrogen source, and thiamine concentration between the two juices as these factors are known to impact the production of acetaldehyde, pyruvic acid, and α-ketoglutarate (Rankine and Pocock, 1969; Rankine, 1967; Rankine, 1965).

While yeast produced different amounts of SO_2 and SO_2 binding compounds, they also influenced the MLF differently. Inhibition of *O. oeni* VFO and the MLF was

dependent on the *S. cerevisiae* strain used to conduct the alcoholic fermentation. In the synthetic grape juice, strains V1116, RUBY.ferm, S102, S325, FX10, F15, and BM45 inhibited the MLF with strong inhibition occurring at the middle of the alcoholic fermentation. Conversely, strains MERIT.ferm, EC1118, S6U, and 43 did not inhibit the MLF. These finding are consistent with other researchers who reported that yeast strains were shown to vary in their antagonism of malolactic bacteria (Osborne and Edwards, 2006; Larsen *et al.,* 2003; Capucho and San Romao, 1994; Dicks *et al*., 1992; Edwards and Beelman, 1987). For example, both Osborne and Edwards (2006) and Larsen *et al*., (2003) reported that V1116 and RUBY.ferm were inhibitory to the MLF. In addition, Osborne and Edwards (2006) also reported that EC1118 was not always inhibitory to the MLF, findings confirmed by this study.

In previous studies, the ability of a yeast strain to inhibit the MLF was attributed to the varied production of antibacterial compounds by the yeast and the sensitivity of bacterial species and strains to these compounds (Osborne and Edwards, 2006; Larsen *et al.,* 2003; Capucho and San Romao, 1994; Henick-Kling and Park, 1994; Dicks *et al*., 1992; Edwards and Beelman, 1987). The most commonly referenced antibacterial compound produced by wine yeast is SO_2 (Osborne and Edwards, 2006; Larsen *et al*., 2003; Carrete *et al*., 2002; Henick-Kling and Park, 1994; Fornachon, 1968). In this study there was some correlation between production of SO_2 with inhibition of MLF occurring in wines containing the highest concentrations of SO_2. For example, MLF was inhibited in juice/wine fermented by V1116, FX10, and BM45 containing high concentrations of SO_2, while MLF was not inhibited in juice/wine fermented by M69 where lower amounts of SO_2 were measured.

Yeast produced SO_2 could not always account for the inhibition of the MLF. For example, during growth in synthetic grape juice yeast strains V1116, MERIT.ferm, and EC1118 statistically produced the same concentration of bound SO_2 yet V1116 was more inhibitory to the MLF. This finding agrees with both Osborne and Edwards (2006) and Larsen *et al.* (2003) where some yeast strains that were inhibitory to MLF did not produce high concentrations of SO_2. In both cases the authors suggested that compounds other than yeast produced SO_2 may have been responsible for MLF inhibition. As further evidence, Osborne and Edwards (2007) reported that the yeast strain RUBY.ferm caused MLF inhibition through the production of an antibacterial peptide. Others have suggested MLF inhibition was caused by lack of nutrients (Henick-Kling, 1993; Kunkee, 1991), or medium chain fatty acids (Edwards and Beelman, 1987).

If yeast produced SO_2 was responsible for MLF inhibition then the inhibition was due to bound SO_2 given that little to no free SO_2 was measured at anytime during the alcoholic fermentations. This finding was observed during fermentations in both synthetic grape juice and Pinot gris grape juice. Other researchers have also reported this with the general conclusion being that bound SO_2 must have some inhibitory action against malolactic bacteria (Osborne and Edwards, 2006; Larsen *et al*., 2003; Henick-Kling and Park, 1994; Eglinton and Henschke, 1996, King and Beelman, 1986; Fornachon, 1963). Fornachon (1963) and Larsen *et al*. (2003) reported inhibition of MLF by yeast produced SO_2 where unidentified bound forms of SO_2 were measured in large amounts while free SO_2 was not detected by either titration or capillary electrophoresis. In addition, a study conducted by Mayer *et al*. (1976) saw similar MLF inhibition as Fornachon (1963) and found an inverse relationship between bacterial growth and the

level of bound SO_2 remaining after fermentation by various yeast strains. Mayer *et al*. (1976) concluded that when more than 50 mg/L bound SO_2 remained after the primary fermentation bacterial growth and MLF were prevented. However, in this present study inhibition was observed in fermentations containing as little as 30 mg/L bound SO_2. This discrepancy may be explained by the media that was being fermented. While Mayer *et al.* (1976) performed studies in grape juice, in this present study inhibition by 30 mg/L bound SO_2 was observed during fermentation of a synthetic grape juice. In fact, when fermentations were performed in Pinot gris juice higher concentrations of bound SO_2 were observed

To date, it has not been demonstrated that SO_2 bound to molecules other than acetaldehyde are toxic to the bacterium. If different forms of bound SO_2 are present at different times during the fermentation then this may account for the varied ability of different yeast strains to inhibit the MLF. However, in this present study acetaldehyde bound SO_2 accounted for all of the bound SO_2 measured at almost all the time points during alcoholic fermentation for all yeast studied. In almost every case, there were always greater concentrations of acetaldehyde present than SO_2 (on a molar basis). Because the binding power of acetaldehyde is so great, in practice no free SO_2 can exist in a wine until all the acetaldehyde is bound. This means that if bound SO_2 was responsible for the MLF inhibition then it was acetaldehyde bound SO_2, a finding in agreement with Fornachon (1963). In contrast, Larsen *et al*. (2003) and Hood (1983) suggested compounds other that acetaldehyde bound to SO_2 were responsible for MLF inhibition. Larsen *et al.* (2003) reported that V1116 inhibited MLF but because of the relative amounts of SO_2 and acetaldehyde measured only a third of the SO_2 was bound to

acetaldehyde. The remaining SO_2 was bound to compounds other than acetaldehyde. However, compared to Larsen *et al.* (2003), a lower concentration of total SO_2 was produced by V1116 during this present study and may account for these conflicting results. For example, Larsen *et al*. (2003), reported a maximum of 75 mg/L SO_2 being produced by V1116 during fermentation in a Chardonnay juice while we observed a maximum of 46 mg/L SO_2 produced during fermentation in a Pinot gris juice. Differences in grape juice composition and fermentation conditions may account for the results reported in these two studies and underline how difficult it can be to compare results from numerous studies where fermentations are conducted under varying conditions and with different grape juices.

The inhibition of the MLF was strongest during the early to mid stages of the alcoholic fermentation in agreement with Larsen *et al*. (2003) who saw strong inhibition during the mid-alcoholic fermentation. Relief of this inhibition was not observed even after 50 days. It has been suggested by other researchers that lees contact can help stimulate the MLF due to either replenishment of nutrients due to yeast autolysis (Patynowski *et al*., 2002; Beelman *et al.,* 1982) or degradation of the toxic compound responsible for the inhibition (Patynowski *et al*., 2002). A potential third reason for the relief of MLF inhibition due to lees contact is a shift in the concentration of the form of bound SO_2 present from an inhibitory form to a less inhibitory form. For example, if acetaldehyde bound SO_2 was more inhibitory to bacteria than pyruvic acid bound SO_2 then, as more pyruvic acid is released into the wine through yeast autolysis, a greater percentage of the bound SO_2 will be pyruvic bound SO_2. This shift may then allow the growth of the bacteria. However, this was not observed in this study. In fact, leaving the

wine in contact with the lees did not result in an increase in pyruvic acid or α-ketoglutarate and so acetaldehyde bound SO_2 was still the dominant form of bound SO_2.

CONCLUSIONS

The present study shows different yeast strains produce a range of SO_2 and SO_2 binding compounds. Yeast that produced the highest levels of SO_2 were most strongly inhibitory to the MLF. Because little to no free SO_2 was measured during the fermentations, inhibition may have been due to bound SO_2. In this study it appears that the bound SO_2 was predominately acetaldehyde bound SO_2 due to the high concentrations of acetaldehyde produced by the yeast. If MLF is to be conducted then the concentration of bound SO_2, as well as free SO_2, need to be considered. However, results also showed that inhibition of MLF did not always correlated to SO_2 production indicating other mechanisms of inhibition. Further work investigating the role of bound SO_2 in causing problematic MLF is required. Studies should utilize a larger number of commercial yeast strains and malolactic bacteria strains. This may help clarify production of SO_2 and SO_2 binding compounds by yeast and possibly yield wines with higher pyruvic acid or α-ketoglutarate bound SO_2 than what was observed in this study. Using different *O. oeni* strains to conduct the MLF may also show different variations of inhibition and resistance to bound SO_2.

CHAPTER 4
Impact of free and bound sulfur dioxide on the growth of wine lactic acid bacteria

ABSTRACT

The impact of free SO_2, acetaldehyde and pyruvic acid along with acetaldehyde bound SO_2, pyruvic acid bound SO_2, and a combinations of acetaldehyde and pyruvic acid bound SO_2 on the growth of wine lactic acid bacteria (LAB) was investigated. In general, most wine LAB did not show signs of growth in media containing only free SO_2 at either pH 3.5 or 3.7 although *Pediococcus damnosus* demonstrated some tolerance to SO_2 with growth in media at pH 3.7 containing up to 20 mg/L free SO_2. Acetaldehyde bound SO_2 appeared to be more inhibitory than pyruvic acid bound SO_2. During growth in media containing acetaldehyde stimulation of growth compared to the control was observed for *Oenococcus oeni* strain VFO and *Lactobacillus hilgardii* at pH 3.5 but not at pH 3.7. Overall, degradation of acetaldehyde and pyruvic acid as well as SO_2 bound acetaldehyde and pyruvic acid was observed for all the LAB tested with the exception of *O. oeni* VFO that did not degrade SO_2 bound pyruvic acid at pH 3.5. Bacteria were inhibited in media containing acetaldehyde bound SO_2 and pyruvic acid bound SO_2 even though degradation of SO_2 bound acetaldehyde and pyruvic acid was observed. This indicates that degradation of the compound bound to SO_2 may have lead to inhibition by the subsequently released free SO_2.

INTRODUCTION

The use of sulfur dioxide (SO_2) is of major importance in winemaking. It acts as both an antioxidant and antimicrobial, and no replacement compound possessing both these properties has been identified. In an aqueous environment SO_2 exists in equilibrium between molecular SO_2, bisulfite ions, and sulfite anions in a pH dependant manner with the dominant species at wine pH (3 to 4) being the bisulfite ion. Besides being in equilibrium with the molecular and sulfite species, bisulfite also exists in either a free or bound form (Fugelsang and Edwards, 2007). Here, the molecule will react with carbonyl compounds such as acetaldehyde forming addition products or adducts such as hydroxysulfonic acids. Although acetaldehyde binds most strongly with SO_2, other carbonyl compounds found in wine, such as pyruvic acid and α-ketoglutaric acid, may also bind with SO_2 (Azevedo *et al.,* 2007; Burroughs and Sparks, 1973; Rankine and Pocock, 1969; Fornachon, 1963).

Free SO_2 (and in particular the molecular portion) is considered to be the most antimicrobial among all the different forms of SO_2 (Rose and Pilkington, 1989; Hinze and Holzer, 1986; Eschenbruch, 1974; Hammond and Carr, 1976). Because molecular SO_2 is not charged it is able to pass across the cell membrane where the cytoplasmic pH (generally near 6.5) cause dissociation to yield bisulfite and sulfite anions. SO_2 inhibits microorganisms primarily by disrupting disulfide bridges in proteins and reacting with cofactors such as NAD^+ and FAD (Fugelsang, 2007). The sensitivity of wine bacteria to SO_2 has been reported by a number of researchers, although there seems to be some ambiguity regarding which wine bacterial species are more sensitive than others. For example, Davis *et al.* (1988) reported that species of *Lactobacillus* and *Pediococcus*

isolated from red wine were more tolerant of higher concentrations of SO_2 than *Oenococcus oeni*. However, Hood (1983) reported that *O. oeni* was more tolerant of SO_2 than the *Pediococcus* and *Lactobacillus* strains tested.

In contrast to free SO_2, bound SO_2 is thought to have much weaker antimicrobial properties. However, a number of researchers have suggested that bound SO_2 may be more antimicrobial than previously believed (Larsen *et al*., 2003; Henick-Kling and Park 1994; Hood, 1983; Fornachon, 1963). For example, Fornachon (1963) found that MLF was prevented in the presence of bound SO_2 where no free SO_2 was measurable. More recently, Larsen *et al*. (2003) and Osborne and Edwards (2006) found a strong correlation between yeast-production of SO_2 and the inhibition of *O. oeni* in both synthetic media and in grape juice. These authors reported that all SO_2 measured was present in unidentified bound forms as free SO_2 was not detected by either titration or capillary electrophoresis analysis. Similar results were also observed by Hood (1983) who suggested a synergistic interaction between bound SO_2 concentrations and pH, with low pH strongly enhancing the antibacterial activity of the bound SO_2.

From these results it is apparent that bound SO_2 possess some antibacterial properties. However there is conflicting information as to which form(s) of bound SO_2 possess the most inhibitory action to wine bacteria. Early work by Fornachon (1963) reported that both *Lb. hilgardii* and *Leuconostoc mesenteriodes* were inhibited in a medium in which SO_2 and an excess of acetaldehyde had been added (thus ensuring that all the SO_2 would be bound to acetaldehyde). The author determined that these bacteria could metabolize acetaldehyde bound SO_2, an observation later confirmed for *O. oeni* (Osborne *et al*., 2006). It was suggested in both studies that the bacterial inhibition was

due to the release of free SO_2 due to the bacterial metabolism of acetaldehyde. In contrast, Carr *et al*. (1976) reported that acetaldehyde bound SO_2 did not have an influence on the bacterium *Lactobacillus plantarum.*

Hood (1983) provided an alternative mechanism by suggesting that any effect of bound SO_2 was due to small amounts of free (specifically molecular) SO_2 in equilibrium with the bound form and also suggested that pyruvic acid bound SO_2 had a greater effect on bacterial growth than acetaldehyde bound SO_2. Work by Larsen *et al*. (2003) helped support Hoods theory that SO_2 bound to compounds other than acetaldehyde can cause LAB inhibition. Larsen *et al*. (2003) showed that *O. oeni* was not inhibited in fermentations containing similar molar concentration of SO_2 and acetaldehyde while strong inhibition was observed in fermentations containing a higher proportion of SO_2 bound to unidentified compounds other than acetaldehyde.

To date, most research on the effects of LAB in the presence of bound SO_2 has focused on acetaldehyde bound SO_2 (Larsen *et al*., 2003; Osborne *et al*., 2000; Hood, 1983; Rankine, 1965). However, it is known that wine can contain high concentrations of SO_2 binding compounds such as pyruvic acid or α-ketoglutaric acid. Little research regarding the effects of SO_2 bound to molecules other than acetaldehyde has been reported. Therefore, the objective of this study was to investigate the impact of acetaldehyde and pyruvic acid bound SO_2 on the growth of wine lactic acid bacteria.

MATERIALS AND METHODS

Microorganisms

The strain of *Oenococcus oeni* used in this study was the freeze-dried form of DSM 7008, Viniflora oenos (Chr. Hansen, Hørsholm, Denmark). In addition, *Pediococcus parvulus* (wine isolate provided by ETS Labs, St. Helena, CA, USA), *P. damnosus* (ATCC 43013), and *Lactobacillus hilgardii* (isolated from a Washington State wine and provided by Dr. Charles Edwards, Washington State University, Pullman, WA, USA) were also used in this study.

P. parvulus, P. damnosus, and *Lb. hilgardii* were maintained in de Man, Rogosa, and Sharpe (MRS) stabs (20g/L Tryptone, 5g/L peptone, 5g/L yeast extract, 5g/L glucose, 200mL apple juice, 1mL Tween 80 [5% w/w solution], 20 g/L agar, pH 4.5) at 4ºC. Freeze-dried *O. oeni* were stored at -20ºC as per manufacturer's instructions.

Starter culture preparation

P. parvulus, P. damnosus, and *Lb. hilgardii* were transferred from the MRS stabs to 250mL MRS broth and incubated aerobically at 25°C for 6 days. Cells were harvested using centrifugation (4,000 x *g* for 20 minutes) and resuspended in 0.2 M phosphate buffer (27.8 g/L $NaH_2PO_4 \cdot H_2O$, 28.38 g/L Na_2HPO_4, pH 7.0) and inoculated at a rate of $1x10^5$ CFU/mL. To prepare the freeze-dried *O. oeni* culture, the bacterium was rehydrated in 0.2M phosphate buffer and directly inoculated at a rate of $1x10^5$ CFU/mL.

Media

A simple LAB growth media based on Hood (1983) was used to measure the impact of bound SO_2 on bacterial growth at wine pH (3 g/L yeast extract, 3 g/L casamino acids, 2 g/L fructose, 6 g/L tartaric acid, 2g/L L-malic acid, 2 g/L K_2HPO_4, 1 g/L $MgSO_4$ $7H_2O$, 20 mg/L $MnSO_4$ H_2O, 20 mg/L $CaCl_2$, 0.5 g/L $FeCl_3$, 1 g/L Tween 80, pH adjusted to 3.5 and 3.7 using NaOH and 25% H_2SO_3). This media was developed to help support the growth of malolactic bacteria while containing very low quantities of compounds that could bind SO_2. Media was sterile filtered through 0.45µm Nalgene PES membrane filter (NalgeNuno International, Rochester, NY, USA) and 9 mL aliquots dispensed into sterile 15 ml screw capped test tubes.

To the growth media, either SO_2, acetaldehyde, pyruvic acid, acetaldehyde bound SO_2, pyruvic acid bound SO_2, or an equilmolar combination of acetaldehyde and pyruvic acid bound SO_2 were added at various concentrations. Solutions were prepared as follows:

Acetaldehyde stock solutions were prepared by adding 1g of fresh acetaldehyde (Sigma Aldrich, St. Louis, MO, USA) (stored cold) into a cold 25mL volumetric flask and cold DI water for a final concentration of 4% (w/v). Pyruvic acid (Sigma Aldrich) stock solutions were prepared by adding 1g of pyruvic acid to 100mL of DI water for a final concentration of 10g/L. A stock SO_2 solution was prepared by adding 0.43g potassium metabisulfite ($K_2S_2O_5$) to 250mL DI water for a final concentration of 500mg/L free SO_2.

To prepare acetaldehyde and pyruvic acid bound SO_2, equimolar amounts of stock acetaldehyde and pyruvic acid solutions were added to SO_2 solutions to give a 1:1 molar

ratio of SO_2:binding compound. Solutions were allowed to equilibrate for 1 to 2 hours. The stock solutions were then diluted so that when 0.1 mLs was added to 9 mLs of growth media, concentrations of 5, 10, 15, 20, 25, or 50 mg/L total SO_2 were achieved. All solutions were sterile filtered using 0.45µm disposable Nalgene PES membrane filter units (NalgeNuno International) before being added to the growth media. Estimated concentrations of SO_2, acetaldehyde, and pyruvic acid for each treatment are shown in Table 4.1. The acetaldehyde and pyruvic acid concentrations used reflect the molar concentrations needed to bind SO_2 in a 1:1 ratio at each corresponding SO_2 concentration. Acetaldehyde and pyruvic acid concentrations were confirmed by enzymatic analysis (enzymatic test kit, R-Biopharm, Darmstadt, Germany), while concentrations of initial free and bound SO_2 were measured by the aeration-oxidation method (Buechsenstein and Ough, 1978). Tubes of growth media containing no addition of SO_2, acetaldehyde, or pyruvic acid were also prepared as controls. All treatments were prepared in triplicate.

Growth Study

Once tubes containing growth media containing either SO_2, acetaldehyde, pyruvic acid, acetaldehyde bound SO_2, pyruvic acid bound SO_2, or acetaldehyde and pyruvic acid bound SO_2 were prepared, they were inoculated with either *O. oeni* VFO, *P. parvulus, P. damnosus,* or *Lb. hilgardii* at approximately 1 x 10^5 CFU/mL. Tubes were incubated at 25°C and growth was followed by measuring changes in optical density at 550 nm using a visible light spectrophotometer (Spectronic 20, Milton Roy Company, Ivyland, PA, USA). Samples (0.5 mL) were also taken on a weekly basis and analyzed for acetaldehyde and pyruvic acid (enzymatic test kit, R-Biopharm).

Table 4.1: Concentrations of SO_2, acetaldehyde, and pyruvic acid present in each treatment (mg/L).

Treatment	SO_2	Acetaldehyde	Pyruvic acid
SO_2	5		
	10		
	15		
	20		
	25		
	50		
Acetaldehyde		8	
		14.5	
		21	
		30	
		36	
		72	
Pyruvic acid			3.5
			7
			10
			15
			18
			36
Bound acetaldehyde	5	8	
	10	14.5	
	15	21	
	20	30	
	25	36	
	50	72	
Bound pyruvic acid	5		3.5
	10		7
	15		10
	20		15
	25		18
	50		36
Bound acetaldehyde & pyruvic acid	5	8	3.5
	10	14.5	7
	15	21	10
	20	30	15
	25	36	18
	50	72	36

RESULTS

Results of the growth studies are summarized in Tables 4.2 to 4.9. Specifically, growth of *O. oeni* VFO, *P. parvulus, P. damnosus,* and *Lb. hilgardii* in media containing various levels of SO_2 showed that at pH 3.5 concentrations as low as 5 mg/L total SO_2 (3.2 mg/L free SO_2) were inhibitory to the growth of the bacterium (Appendix E, G, I, K). At pH 3.7, *O. oeni* VFO, *P. parvulus*, and *Lb. hilgardii* were still inhibited (Appendix F, H, L). However, *P. damnosus* was able to grow in media containing free SO_2. For example as displayed in Figure 4.1, at pH 3.7 *O. oeni VFO* did not show signs of growth even when the total SO_2 (3.7 mg/L free SO_2) concentration was very low. In contrast, *P. damnosus* exhibited signs of growth reaching a maximum OD reading of around 0.60 even when 25 mg/L SO_2 (18.4 mg/L free SO_2) was present in the media (Figure 4.1B).

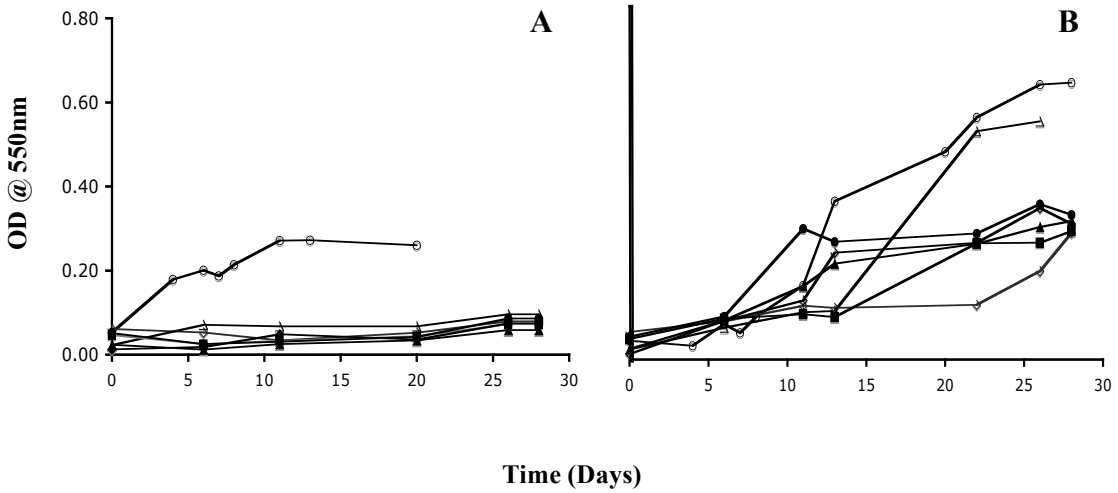


Figure 4.1: Growth of *O. oeni* VFO (**A**) and *P. damnosus* (**B**) in Hood Media at pH 3.7 containing total SO_2 at 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇. Results are means of triplicate samples.

Prior to bacterial inoculation into media containing only SO_2, concentrations of free and bound SO_2 were confirmed (Appendix A). Results showed that when SO_2 concentrations of 5, 10, 15, 20, 25, and 50 mg/L were added, a majority of the SO_2 was present as free SO_2. However due to the nature of the media and SO_2 having a small affinity for sugar compounds (such as fructose) a small portion of the total SO_2 was present as bound SO_2 at both pH 3.5 and 3.7 (Appendix A).

Growth studies investigating the inhibitory effect of bound SO_2 were carried out in the same manner as the free SO_2 experiments. Media spiked with acetaldehyde bound SO_2 was allowed to equilibrate and free and bound SO_2 concentrations were measured (Appendix B). Results confirm that all of the SO_2 added to the media was in the form of bound SO_2 with little to no free SO_2 being present at almost all the concentrations tested at both pH 3.5 and 3.7. At pH 3.5, *O. oeni* VFO*, P. parvulus,* and *P. damnosus* did not exhibit signs of growth in the presence of acetaldehyde bound SO_2 (Figure 4.2) at any of the concentrations tested. However, an increase in OD was observed for *Lb. hilgardii* by day 20 but growth was much lower compared to the control containing no acetaldehyde bound SO_2 (Figure 4.2D).

However when the bacteria were inoculated into media at pH 3.7 all the bacterial species were less inhibited by the acetaldehyde bound SO_2 (Figure 4.3). *O. oeni* VFO was able to grow when the acetaldehyde bound SO_2 concentration was 5 mg/L but not able to grow at higher concentrations. *P. damnosus* also displayed signs of growth at pH 3.7 and was able to grow when 5 mg/L of acetaldehyde bound SO_2 was present in the system (Figure 4.3C).

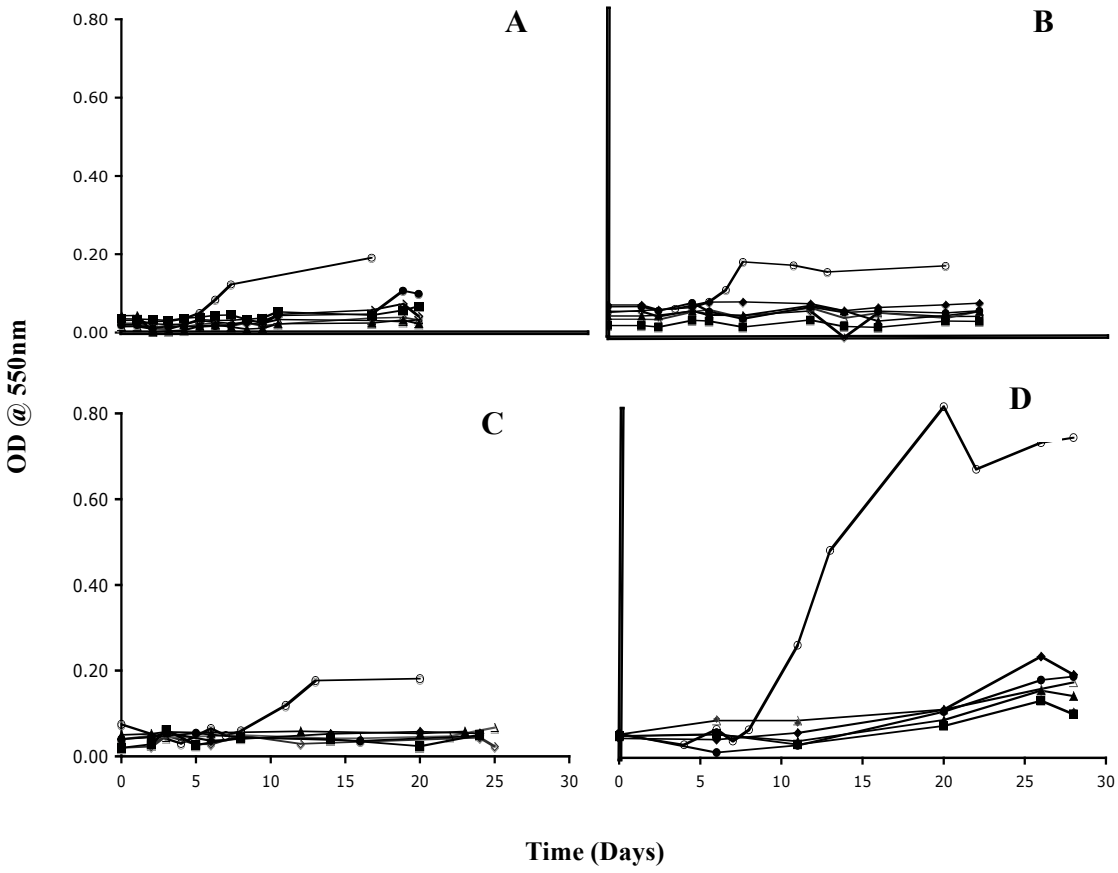


Figure 4.2: Growth of *O. oeni* VFO (**A**), *P. parvulus* (**B**), *P. damnosus* (**C**), and *Lb. hilgardii* (**D**) in Hood Media at pH 3.5 containing acetaldehyde bound SO_2 at 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇. Results are means of triplicate samples.

It is also interesting to note that at concentrations of 10, 15, 20, and 25 mg/L *P. damnosus* also grew after a lag phase of around 10 days. In contrast, *P. parvulus* and *Lb. hilgardii* did not show signs of growth at pH 3.7 for any of concentrations of acetaldehyde bound SO_2 tested (Figure 4.3B & D).

At various time points during the growth study samples were taken and measured for acetaldehyde. Degradation of acetaldehyde in the acetaldehyde bound SO_2 studies was observed for all LAB strains at pH 3.5 and 3.7 (Appendix O, U, AA, GG). It is interesting to note that at pH 3.5, acetaldehyde concentrations did not decrease as fast as

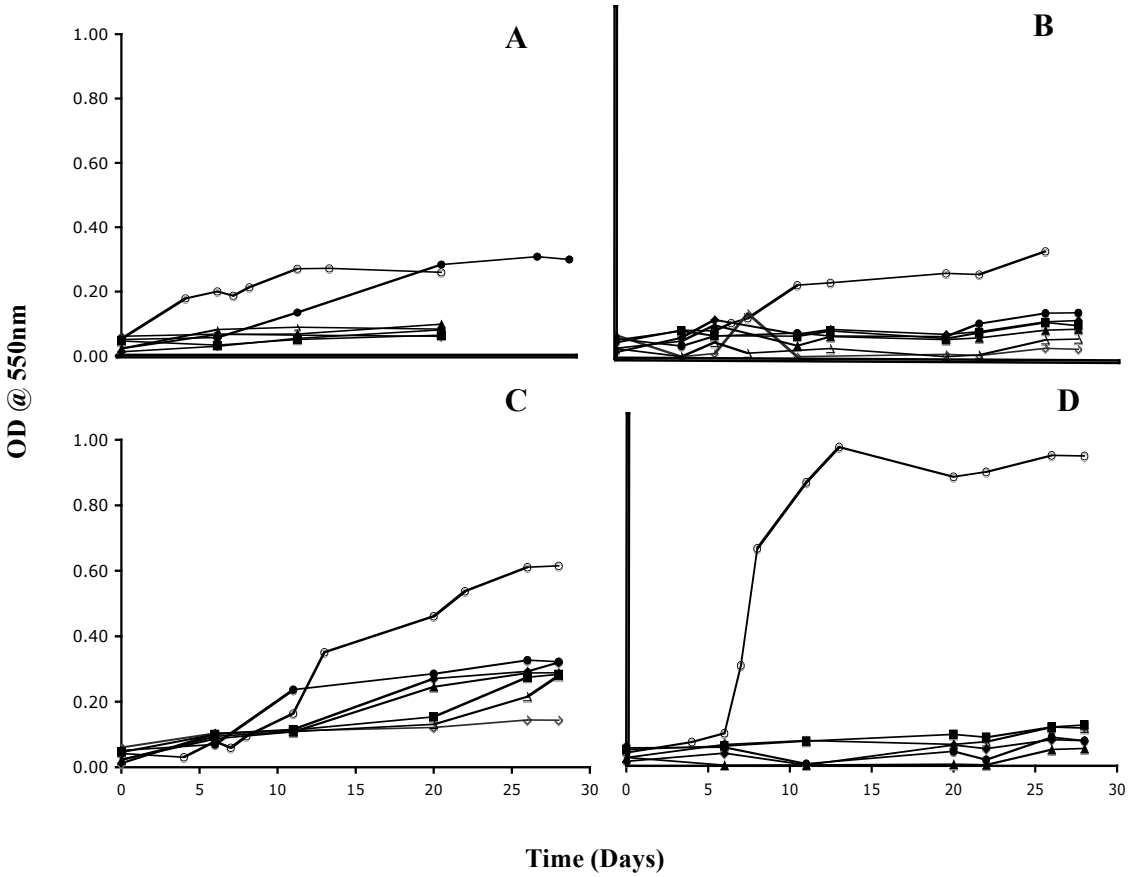


Figure 4.3: Growth of *O. oeni* VFO (**A**), *P. parvulus* (**B**), *P. damnosus* (**C**), and *Lb. hilgardii* (**D**) in Hood Media at pH 3.7 containing acetaldehyde bound SO_2 at 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇. Results are means of triplicate samples.

at pH of 3.7 (Appendix O, U, AA, GG). However, by the first sampling day in media at pH 3.7 almost all of the acetaldehyde had been degraded and by the end of the experiment no acetaldehyde was measured in media inoculated with *O. oeni* VFO, *P. parvulus*, *P. damnosus*, or *Lb. hilgardii*.

Besides observing the effects of SO_2 and acetaldehyde bound SO_2, the effect of pyruvic acid bound SO_2 was also examined. Bound and free SO_2 was measured in the pyruvic acid bound SO_2 solutions prior to inoculation (Appendix C). A proportion of the SO_2 was always present as free SO_2 even though pyruvic acid and SO_2 were mixed in

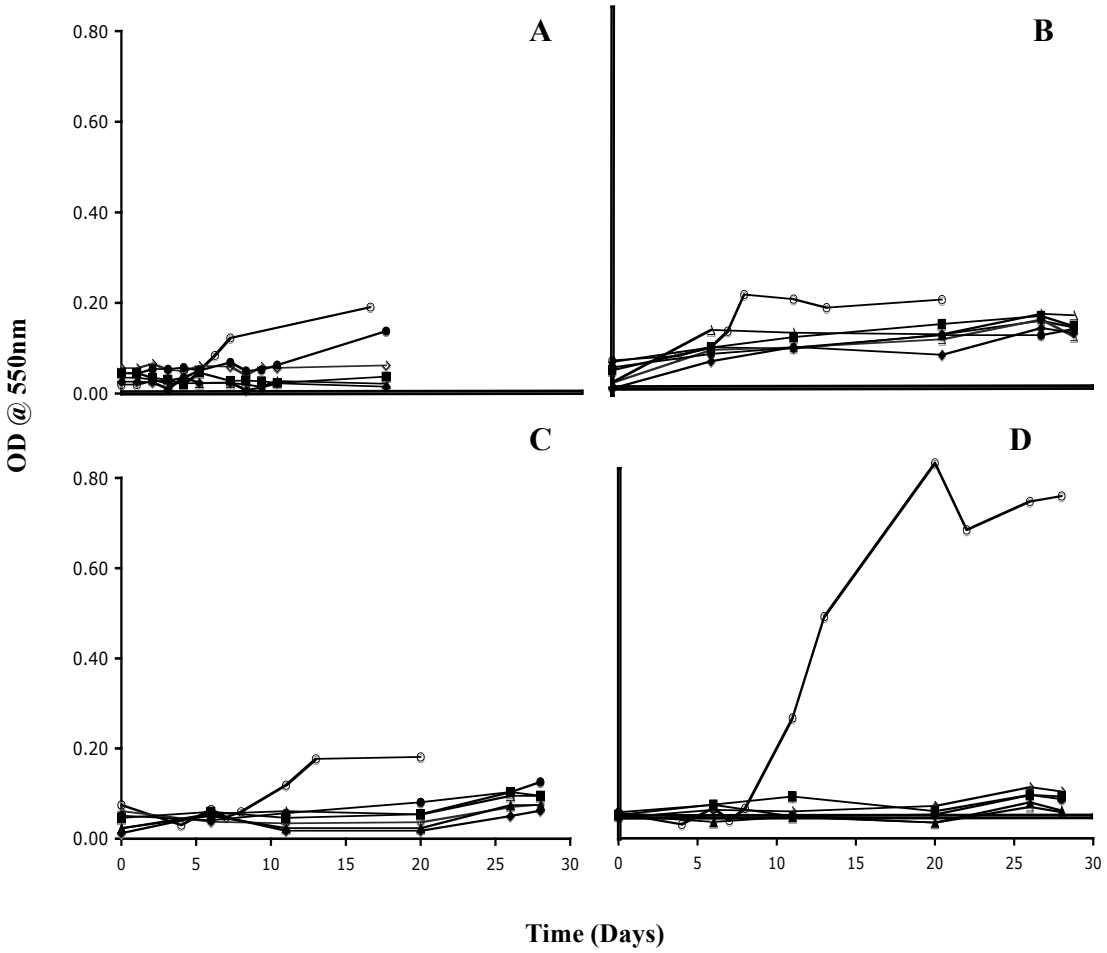


Figure 4.4: Growth of *O. oeni* VFO (**A**), *P. parvulus* (**B**), *P. damnosus* (**C**), and *Lb. hilgardii* (**D**) at 550nm in Hood Media at pH 3.5 containing pyruvic acid bound SO_2 at 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇. Results are means of triplicate samples.

equimolar concentrations. A ratio of about 1:2 (free SO_2: pyruvic acid bound SO_2) existed when only pyruvic acid was added to a SO_2 solution (Appendix C).

As displayed in Figure 4.4, pyruvic acid bound SO_2 was inhibitory to the growth of all four LAB tested at pH 3.5. However, the effects of pyruvic acid bound SO_2 did not appear to be as inhibitory as the acetaldehyde bound SO_2 treatments. An increase in OD was observed for *O. oeni* VFO near the end of the growth study in media containing 5 mg/L pyruvic acid bound SO_2 (Figure 4.4A).

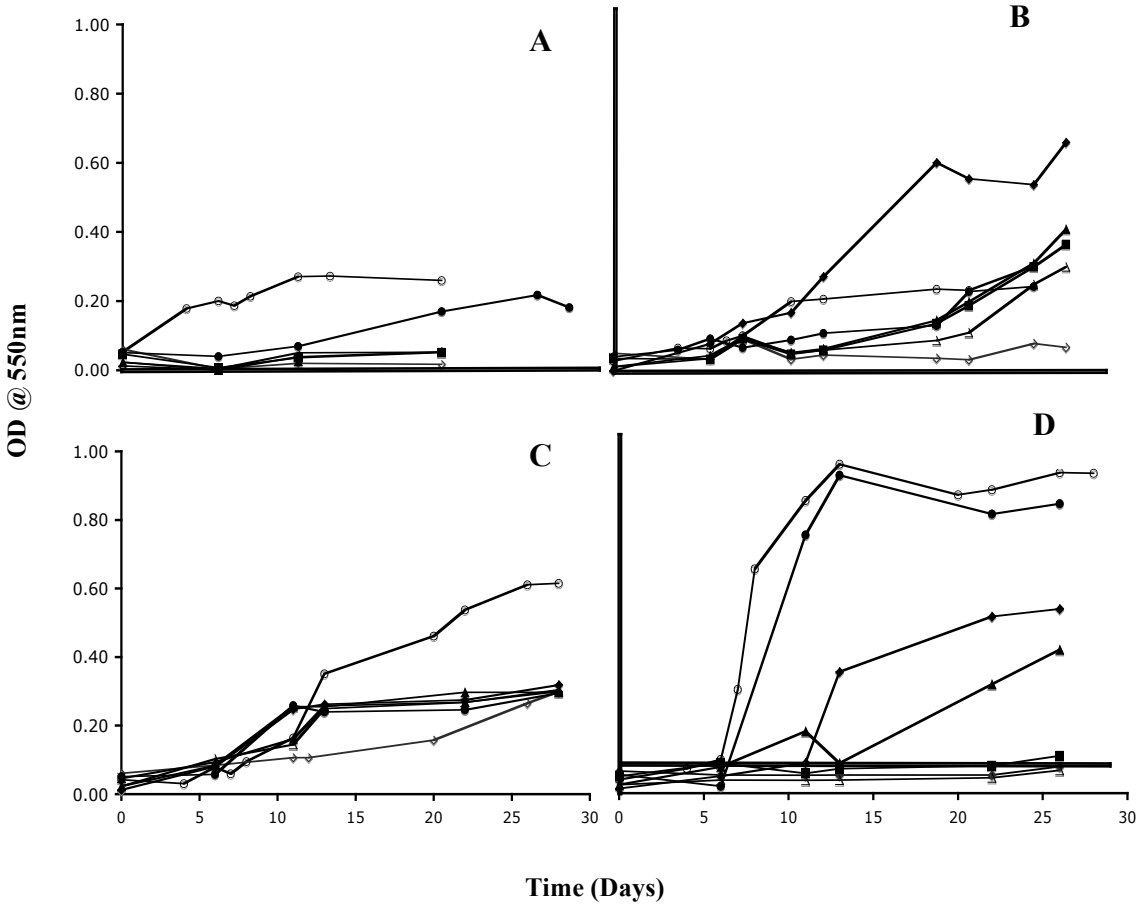


Figure 4.5: Growth of *O. oeni* VFO (**A**), *P. parvulus* (**B**), *P. damnosus* (**C**), and *Lb. hilgardii* (**D**) in Hood Media at pH 3.7 containing pyruvic acid bound SO_2 at 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇. Results are means of triplicate samples.

In addition, *P. parvulus* and *P. damnosus* exhibited slight growth in media containing pyruvic acid bound SO_2 (Figure 4.4B & C).

Pyruvic acid bound SO_2 was not as inhibitory to bacterial growth at pH 3.7 when compared to 3.5. For example, at pH 3.7 *O. oeni* VFO showed growth when 5 mg/L of pyruvic acid bound SO_2 was present but not at 10, 15, 20 15, or 50 mg/L (Figure 4.5A). *P. parvulus* initially exhibited delayed growth but after 10 days increases in OD were observed with OD values being higher in tubes containing 10 mg/L pyruvic acid bound

SO_2 then in the control (Figure 4.5B). *P. damnosus* also displayed increases in OD but not

as much as the control (Figure 4.5C). Finally, *Lb. hilgardii* showed large increases in OD in media containing 5, 10, and 15 mg/L pyruvic acid bound SO_2 with growth at 5 mg/L pyruvic acid bound SO_2 being very similar to that of the control (Figure 4.5D).

At various time points during the experiment, samples were removed and tested for pyruvic acid. After each sampling day, a reduction in pyruvic acid in the pyruvic acid bound SO_2 treatments was observed for *O. oeni* VFO*, P. parvulus, P. damnosus,* and *Lb. hilgardii* at pH 3.5 and 3.7, with the exception of *O. oeni* at pH 3.5 where no reduction of pyruvic acid was observed at any sampling day (Appendix P, V, BB, HH). The decrease in measured pyruvic acid was most significant after the first sampling day.

Finally, the media was spiked with a combination of acetaldehyde bound SO_2 and pyruvic acid bound SO_2 and allowed to equilibrate before free and bound SO_2 were measured (Appendix D). Results demonstrated that no free SO_2 was measured at any of the concentrations indicating that all the SO_2 added was present as bound SO_2. At pH 3.5, no growth was observed for any of the bacteria (Figure 4.6). A slight increase in OD was observed for *O. oeni* VFO when the concentration of acetaldehyde and pyruvic acid bound SO_2 was 5 mg/L (Figure 4.6A). However, no growth was observed at higher concentrations.

At pH 3.7, *O. oeni* VFO displayed growth only at the 5 mg/L concentration (Figure 4.7A). *P. parvulus* showed growth after a delay of 20 days in media containing 5, 10, and 15 mg/L acetaldehyde and pyruvic acid bound SO_2 but not at higher concentrations

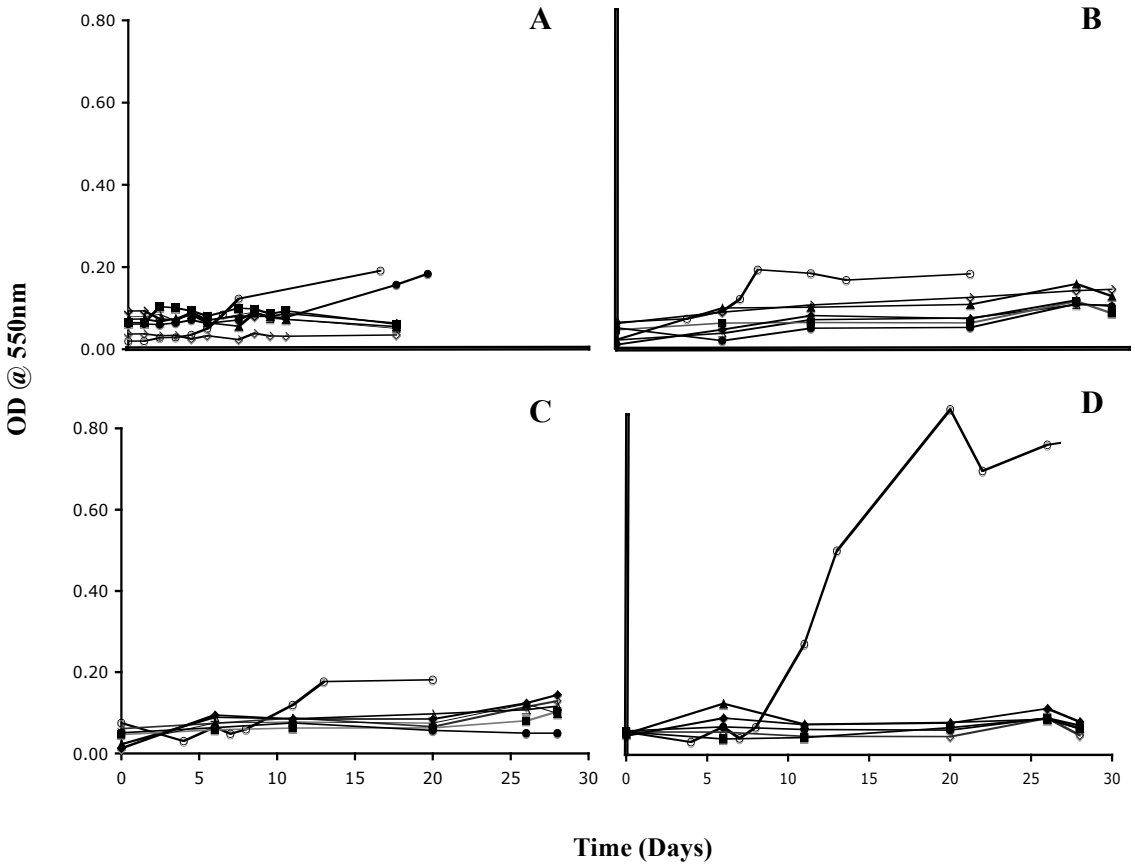


Figure 4.6: Growth of *O. oeni* VFO (**A**), *P. parvulus* (**B**), *P. damnosus* (**C**), and *Lb. hilgardii* (**D**) in Hood Media at pH 3.5 containing acetaldehyde and pyruvic acid bound SO_2 at 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇. Results are means of triplicate samples.

(Figure 4.7B). *P. damnosus* showed an increase in OD at all concentrations of acetaldehyde and pyruvic acid bound SO_2 but growth at 25 and 50 mg/L was delayed (Figure 4.7C) while *Lb. hilgardii* was able to grow when 5 mg/L of acetaldehyde and pyruvic acid bound SO_2 was added to the media (Figure 4.7D).

In media containing both acetaldehyde and pyruvic acid bound SO_2, acetaldehyde and pyruvic acid were degraded by *P. parvulus, P. damnosus,* and *Lb. hilgardii* at pH 3.5 and 3.7 (Appendix W, X, CC, DD, II, JJ), while *O. oeni* VFO completely degraded acetaldehyde and pyruvic acid at pH 3.7 (Appendix R). but only small amounts of

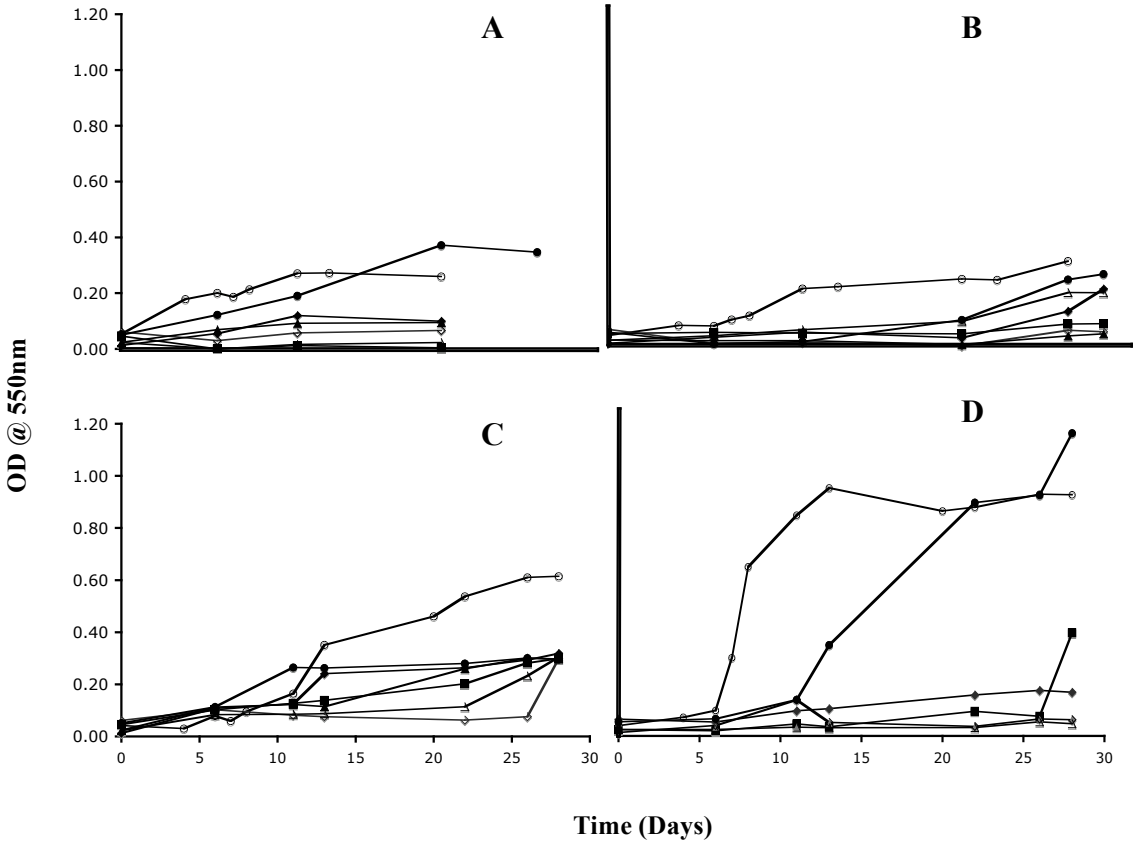


Figure 4.7: Growth of *O. oeni* VFO (**A**), *P. parvulus* (**B**), *P. damnosus* (**C**), and *Lb. hilgardii* (**D**) in Hood Media at pH 3.7 containing acetaldehyde and pyruvic acid bound SO_2 at 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇. Results are means of triplicate samples.

acetaldehyde was reduced at pH 3.5 with no reduction in pyruvic acid (Appendix Q). Reduction in both acetaldehyde and pyruvic acid occurred between day 0 and day 7 for *P. parvulus*, *P. damnosus*, and *Lb. hilgardii* at pH 3.5 and 3.7; however by the second sampling day no pyruvic acid was measured in any concentration at either pH 3.5 and 3.7.

In contrast to the observed inhibition of bacterial growth in media containing free or bound SO_2, stimulation of growth in the presence of acetaldehyde was observed for *O. oeni* and *Lb. hilgardii* at pH 3.5 (Figure 4.8A & D). Compared to the control, *O. oeni* VFO grew more quickly in media containing acetaldehyde (Figure 4.8A).

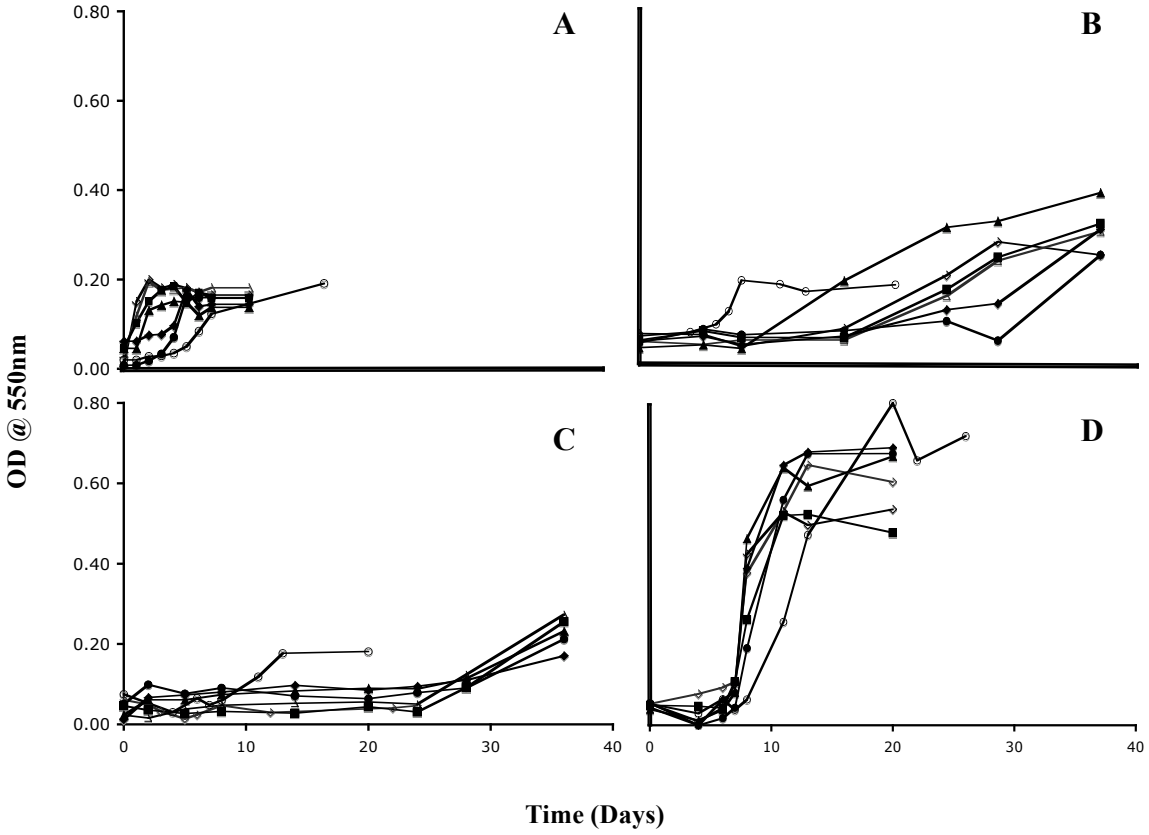


Figure 4.8: Growth of *O. oeni* VFO (**A**), *P. parvulus* (**B**), *P. damnosus* (**C**), and *Lb. hilgardii* (**D**) in Hood Media at pH 3.5 containing acetaldehyde at 0mg/L ○; 8 mg/L ●; 14.5 mg/L ◆; 21 mg/L ▲; 30 mg/L ■; 36 mg/L △; or 72 mg/L ◇. Results are means of triplicate samples.

For example, in media containing 72 mg/L acetaldehyde an OD value of 0.2 was observed after 3 days versus 16 days for the control. This was also the case for *Lb. hilgardii* (Figure 4.8D) where earlier increases in OD were observed in media containing acetaldehyde.

In contrast, acetaldehyde appeared to delay the growth of both *Pediococcus* species with increases in OD not observed until around day 15 (*P. parvulus*) or day 25 (*P. damnosus*) (Figure 4.8B & C). However growth by *P. parvulus* appeared to be

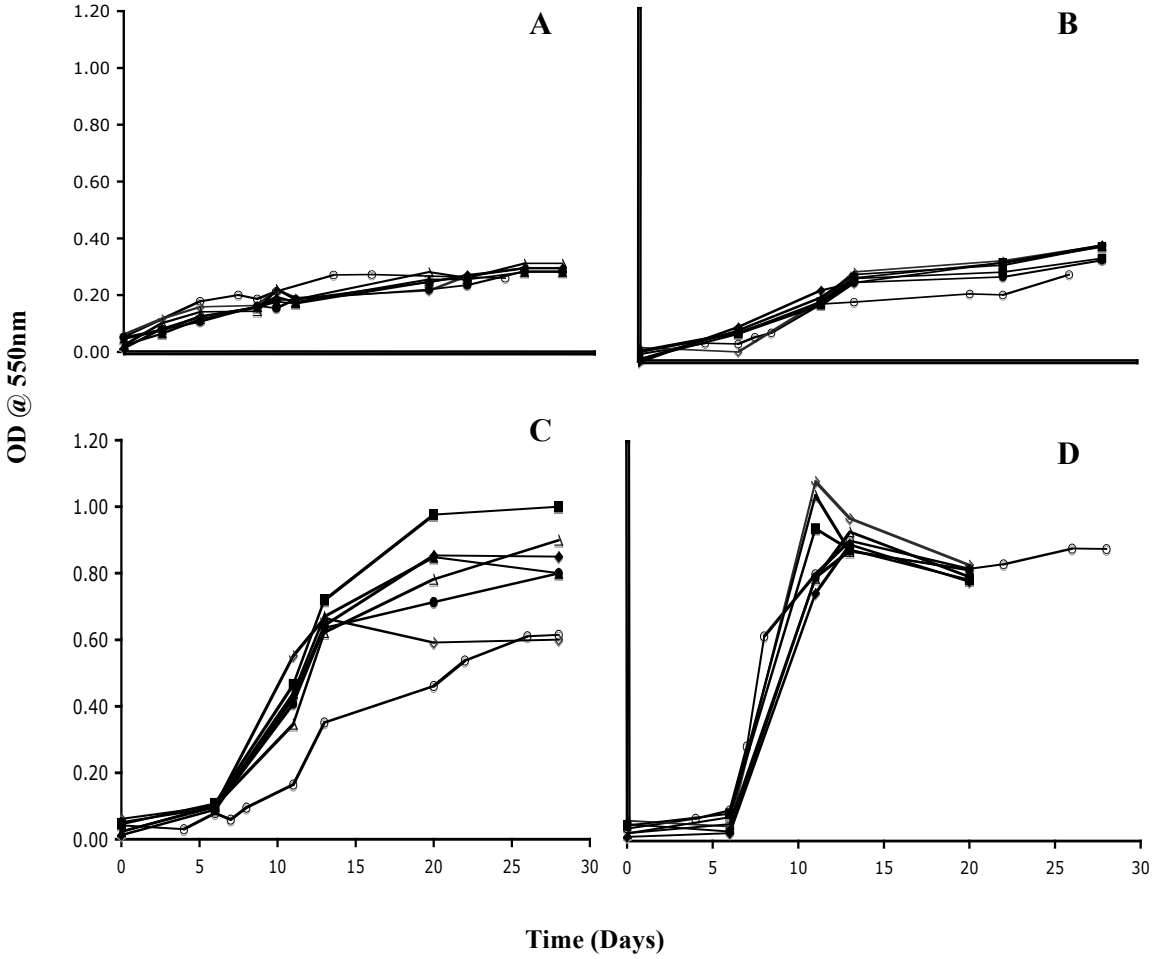


Figure 4.9: Growth of *O. oeni* VFO (**A**), *P. parvulus* (**B**), *P. damnosus* (**C**), and *Lb. hilgardii* (**D**) in Hood Media at pH 3.7 containing acetaldehyde at 0 mg/L ○; 8 mg/L ●; 14.5 mg/L ◆; 21 mg/L ▲; 30 mg/L ■; 36 mg/L △; or 72 mg/L ◇. Results are means of triplicate samples.

stimulatory at 30 mg/L acetaldehyde in that a higher final OD value was obtained compared to the control (Figure 4.8B).

In contrast, at pH 3.7 acetaldehyde did not appear to stimulate the growth of *O. oeni* VFO, *P. parvulus*, and *Lb. hilgardii* (Figure 4.9A, B & D). However, acetaldehyde did appear to stimulate the growth of *P. damnosus* as OD values increased earlier than the control and reached a higher final value (Figure 4.9C). For all treatments little to no acetaldehyde was measured after bacterial growth in the media for 30 days with the majority of the acetaldehyde degraded after 7 days (Appendix M, S, Y, EE).

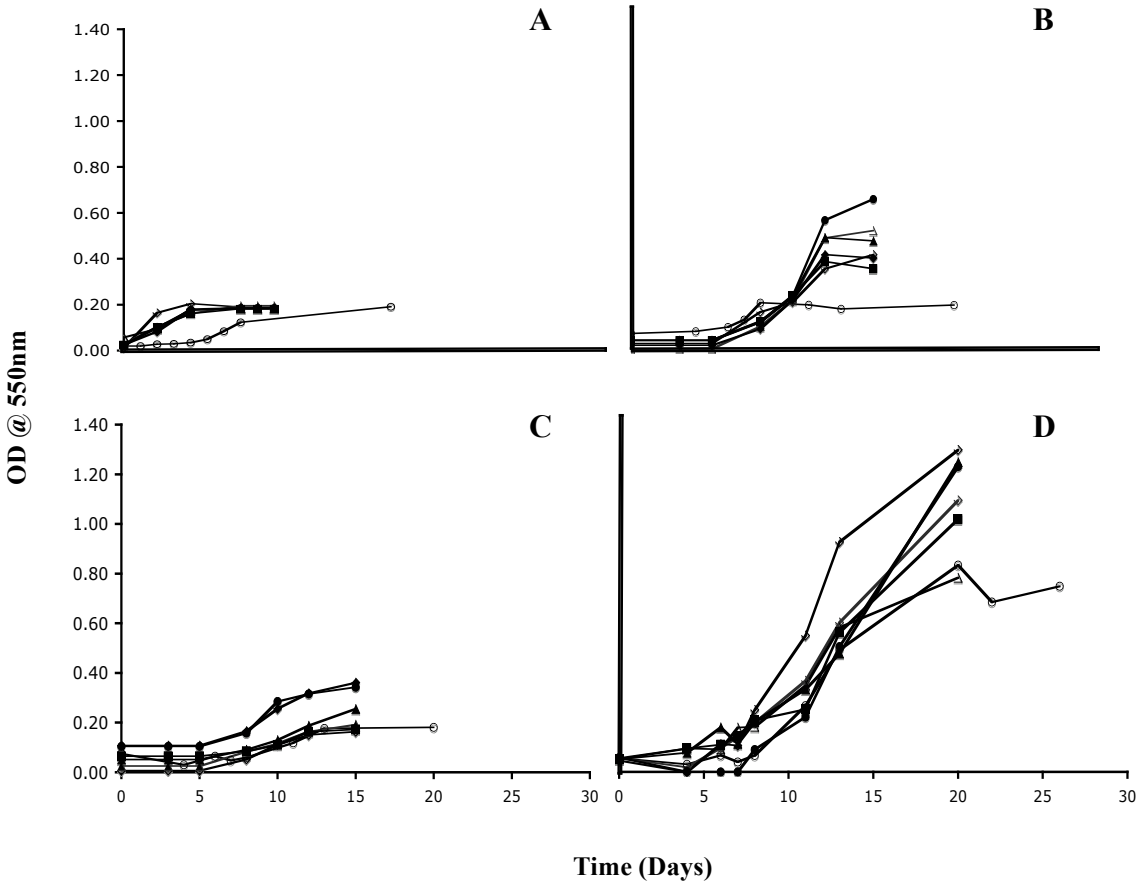


Figure 4.10: Growth of *O. oeni* VFO (**A**), *P. parvulus* (**B**), *P. damnosus* (**C**), and *Lb. hilgardii* (**D**) in Hood Media at pH 3.5 containing pyruvic acid at 0mg/L ○; 3.5 mg/L ●; 7 mg/L ◆; 10 mg/L ▲; 15 mg/L ■; 18 mg/L △; or 36 mg/L ◇. Results are means of triplicate samples.

However, at pH 3.5 both *Pediococcus* strains took longer to degrade the acetaldehyde (36 days) as compared to *O. oeni* VFO and *Lb. hilgardii* at pH 3.5 (14 days) (Appendix M, S, Y, EE). Yet at pH 3.7, both *Pediococcus* strains degraded acetaldehyde more quickly (14 day or 28 days) than at pH 3.5 (Appendix S, Y).

In media containing pyruvic acid growth of *O. oeni* VFO was stimulated at pH 3.5 (Figure 4.10A) with the maximum OD readings of 0.2 occurring 5 days following inoculation. Stimulated growth was also observed for strains *P. parvulus*, *P. damnosus,*

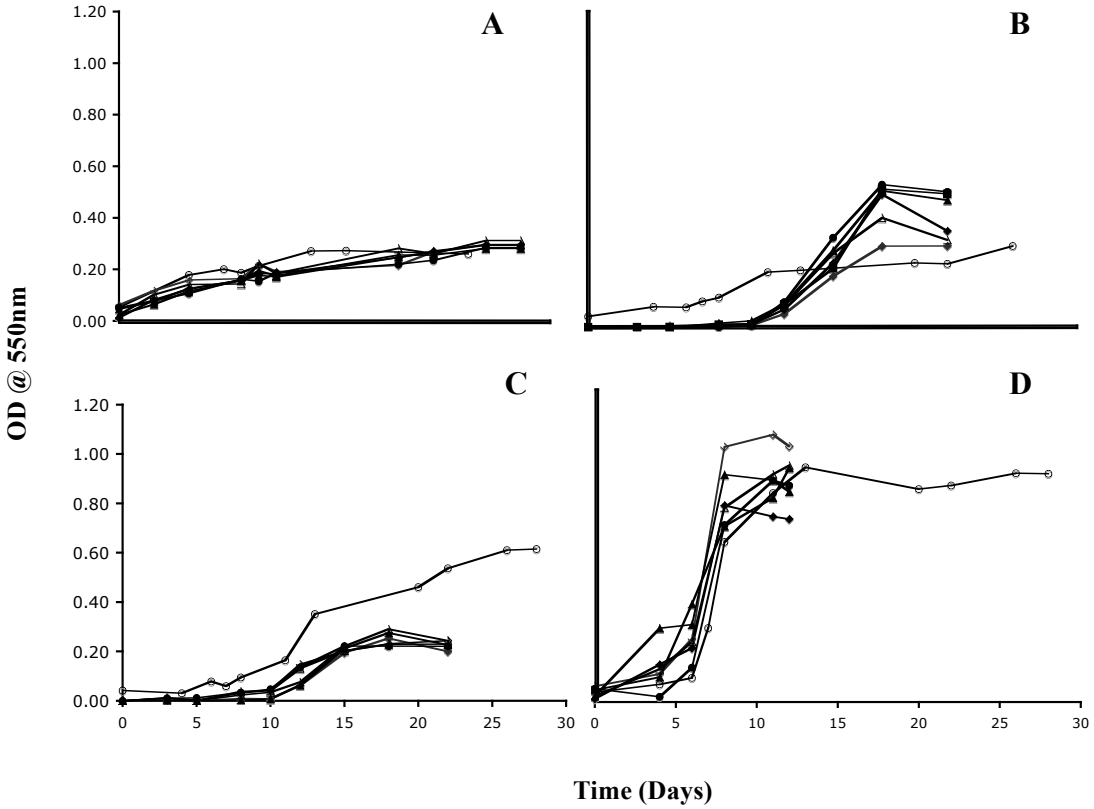


Figure 4.11: Growth of *O. oeni* VFO (**A**), *P. parvulus* (**B**), *P. damnosus* (**C**), and *Lb. hilgardii* (**D**) in Hood Media at pH 3.7 containing pyruvic acid at 0mg/L ○; 3.5 mg/L ●; 7 mg/L ◆; 10 mg/L ▲; 15 mg/L ■; 18 mg/L △; or 36 mg/L ◇. Results are means of triplicate samples.

and *Lb. hilgardii* at pH 3.5 (Figure 4.10B, C & D) with much higher final OD values being observed in media containing pyruvic acid.

At pH 3.7, growth of *O. oeni* VFO and *Lb. hilgardii* in media containing pyruvic acid was similar to the control even with the addition of pyruvic acid to the media (Figure 4.11A & D). In contrast, *P. parvulus* and *P. damnosus* initially displayed delayed growth although in the case of *P. parvulus*, a higher final OD reading was observed during growth in media containing pyruvic acid (Figure 4.11B & C). In all treatments, pyruvic acid was degraded by *O. oeni* VFO, *P. parvulus*, *P. damnosus,* and *Lb. hilgardii* with very little pyruvic acid being present after 7 days growth of the bacteria (Appendix N, T, Z, FF).

Table 4.2: Summary of the growth response of *O. oeni* VFO in Hood media at pH 3.5 containing either SO_2, acetaldehyde, pyruvic acid, acetaldehyde bound SO_2, pyruvic acid bound SO_2, or acetaldehyde and pyruvic acid bound SO_2 and whether or not degradation of acetaldehyde or pyruvic acid occurred.

Treatment	SO_2 (mg/L)	Acetaldehyde (mg/L)	Pyruvic acid (mg/L)	Growth response	Degradation
SO_2	5			- - -	n/a
	10			- - -	n/a
	15			- - -	n/a
	20			- - -	n/a
	25			- - -	n/a
	50			- - -	n/a
Acetaldehyde		8		+ + +	Yes
		14.5		+ + +	Yes
		21		+ + +	Yes
		30		+ + +	Yes
		36		+ + +	Yes
		72		+ + +	Yes
Pyruvic acid			3.5	+ + +	Yes
			7	+ + +	Yes
			10	+ + +	Yes
			15	+ + +	Yes
			18	+ + +	Yes
			36	+ + +	Yes
Bound acetaldehyde	5	8		- - -	Yes
	10	14.5		- - -	Yes
	15	21		- - -	Yes
	20	30		- - -	Yes
	25	36		- - -	Yes
	50	72		- - -	Yes
Bound pyruvic acid	5		3.5	- -	No
	10		7	- - -	No
	15		10	- - -	No
	20		15	- - -	No
	25		18	- - -	No
	50		36	- - -	No
Bound acetaldehyde, pyruvic acid	5	8	3.5	- -	Yes/No[1]
	10	14.5	7	- - -	Yes/No[1]
	15	21	10	- - -	Yes/No[1]
	20	30	15	- - -	Yes/No[1]
	25	36	18	- - -	Yes/No[1]
	50	72	36	- - -	Yes/No[1]

[1]Acetaldehyde degraded but not pyruvic acid

- - - Very delayed growth as compared to control; No growth in 28days
- - Delayed growth as compared to control; 10 days ≤ Growth ≤ 28 days
- Slightly delayed growth as compared to control; Growth ≤ 10 days
+/- Growth same as control
+ Slight stimulation of growth as compared to the control
+ + Stimulated growth as compared to the control
+ + + Strongly stimulated growth as compared to control; growth > control

Table 4.3: Summary of the growth response of *O. oeni* VFO in Hood media at pH 3.7 containing either SO_2, acetaldehyde, pyruvic acid, acetaldehyde bound SO_2, pyruvic acid bound SO_2, or acetaldehyde and pyruvic acid bound SO_2 and whether or not degradation of acetaldehyde or pyruvic acid occurred.

Treatment	SO_2 (mg/L)	Acetaldehyde (mg/L)	Pyruvic acid (mg/L)	Growth response	Degradation
SO_2	5			- - -	n/a
	10			- - -	n/a
	15			- - -	n/a
	20			- - -	n/a
	25			- - -	n/a
	50			- - -	n/a
Acetaldehyde		8		+ /-	Yes
		14.5		+ /-	Yes
		21		+ /-	Yes
		30		+ /-	Yes
		36		+ /-	Yes
		72		+ /-	Yes
Pyruvic acid			3.5	+ /-	Yes
			7	+ /-	Yes
			10	+ /-	Yes
			15	+ /-	Yes
			18	+ /-	Yes
			36	+ /-	Yes
Bound acetaldehyde	5	8		- -	Yes
	10	14.5		- - -	Yes
	15	21		- - -	Yes
	20	30		- - -	Yes
	25	36		- - -	Yes
	50	72		- - -	Yes
Bound pyruvic acid	5		3.5	- -	Yes
	10		7	- - -	Yes
	15		10	- - -	Yes
	20		15	- - -	Yes
	25		18	- - -	Yes
	50		36	- - -	Yes
Bound acetaldehyde, pyruvic acid	5	8	3.5	-	Yes
	10	14.5	7	- - -	Yes
	15	21	10	- - -	Yes
	20	30	15	- - -	Yes
	25	36	18	- - -	Yes
	50	72	36	- - -	Yes

- - - Very delayed growth as compared to control; No growth in 28days
- - Delayed growth as compared to control; 10 days ≤ Growth ≤ 28 days
- Slightly delayed growth as compared to control; Growth ≤ 10 days
+/- Growth same as control
+ Slight stimulation of growth as compared to the control
+ + Stimulated growth as compared to the control
+ + + Strongly stimulated growth as compared to control; growth > control

Table 4.4: Summary of the growth response of *P. parvulus* in Hood media at pH 3.5 containing either SO_2, acetaldehyde, pyruvic acid, acetaldehyde bound SO_2, pyruvic acid bound SO_2, or acetaldehyde and pyruvic acid bound SO_2 and whether or not degradation of acetaldehyde or pyruvic acid occurred.

Treatment	SO_2 (mg/L)	Acetaldehyde (mg/L)	Pyruvic acid (mg/L)	Growth response	Degradation
SO_2	5			- - -	n/a
	10			- - -	n/a
	15			- - -	n/a
	20			- - -	n/a
	25			- - -	n/a
	50			- - -	n/a
Acetaldehyde		8		-	Yes
		14.5		- -	Yes
		21		- -	Yes
		30		- -	Yes
		36		- -	Yes
		72		- -	Yes
Pyruvic acid			3.5	+ + +	Yes
			7	+ + +	Yes
			10	+ + +	Yes
			15	+ + +	Yes
			18	+ + +	Yes
			36	+ + +	Yes
Bound acetaldehyde	5	8		- - -	Yes
	10	14.5		- - -	Yes
	15	21		- - -	Yes
	20	30		- - -	Yes
	25	36		- - -	Yes
	50	72		- - -	Yes
Bound pyruvic acid	5		3.5	- -	Yes
	10		7	- -	Yes
	15		10	- -	Yes
	20		15	- -	Yes
	25		18	- -	Yes
	50		36	- -	Yes
Bound acetaldehyde, pyruvic acid	5	8	3.5	- - -	Yes
	10	14.5	7	- - -	Yes
	15	21	10	- - -	Yes
	20	30	15	- - -	Yes
	25	36	18	- - -	Yes
	50	72	36	- - -	Yes

- - - Very delayed growth as compared to control; No growth in 28days
- - Delayed growth as compared to control; 10 days ≤ Growth ≤ 28 days
- Slightly delayed growth as compared to control; Growth ≤ 10 days
+/- Growth same as control
+ Slight stimulation of growth as compared to the control
+ + Stimulated growth as compared to the control
+ + + Strongly stimulated growth as compared to control; growth > control

Table 4.5: Summary of the growth response of *P. parvulus* in Hood media at pH 3.7 containing either SO_2, acetaldehyde, pyruvic acid, acetaldehyde bound SO_2, pyruvic acid bound SO_2, or acetaldehyde and pyruvic acid bound SO_2 and whether or not degradation of acetaldehyde or pyruvic acid occurred.

Treatment	SO_2 (mg/L)	Acetaldehyde (mg/L)	Pyruvic acid (mg/L)	Growth response	Degradation
SO_2	5			- - -	n/a
	10			- - -	n/a
	15			- - -	n/a
	20			- - -	n/a
	25			- - -	n/a
	50			- - -	n/a
Acetaldehyde		8		+ / -	Yes
		14.5		+ / -	Yes
		21		+ / -	Yes
		30		+ / -	Yes
		36		+ / -	Yes
		72		+ / -	Yes
Pyruvic acid			3.5	-	Yes
			7	-	Yes
			10	-	Yes
			15	-	Yes
			18	-	Yes
			36	-	Yes
Bound acetaldehyde	5	8		- - -	Yes
	10	14.5		- - -	Yes
	15	21		- - -	Yes
	20	30		- - -	Yes
	25	36		- - -	Yes
	50	72		- - -	Yes
Bound pyruvic acid	5		3.5	-	Yes
	10		7	-	Yes
	15		10	-	Yes
	20		15	-	Yes
	25		18	-	Yes
	50		36	-	Yes
Bound acetaldehyde, pyruvic acid	5	8	3.5	- -	Yes
	10	14.5	7	- -	Yes
	15	21	10	- -	Yes
	20	30	15	- -	Yes
	25	36	18	- -	Yes
	50	72	36	- -	Yes

- - - Very delayed growth as compared to control; No growth in 28days
- - Delayed growth as compared to control; 10 days ≤ Growth ≤ 28 days
- Slightly delayed growth as compared to control; Growth ≤ 10 days
+/- Growth same as control
+ Slight stimulation of growth as compared to the control
+ + Stimulated growth as compared to the control
+ + + Strongly stimulated growth as compared to control; growth > control

Table 4.6: Summary of the growth response of *P. damnosus* in Hood media at pH 3.5 containing either SO_2, acetaldehyde, pyruvic acid, acetaldehyde bound SO_2, pyruvic acid bound SO_2, or acetaldehyde and pyruvic acid bound SO_2 and whether or not degradation of acetaldehyde or pyruvic acid occurred.

Treatment	SO_2 (mg/L)	Acetaldehyde (mg/L)	Pyruvic acid (mg/L)	Growth response	Degradation
SO_2	5			- - -	n/a
	10			- - -	n/a
	15			- - -	n/a
	20			- - -	n/a
	25			- - -	n/a
	50			- - -	n/a
Acetaldehyde		8		- -	Yes
		14.5		- -	Yes
		21		- -	Yes
		30		- -	Yes
		36		- -	Yes
		72		- -	Yes
Pyruvic acid			3.5	+ + +	Yes
			7	+ + +	Yes
			10	+ +	Yes
			15	+ / -	Yes
			18	+ / -	Yes
			36	+ / -	Yes
Bound acetaldehyde	5	8		- - -	Yes
	10	14.5		- - -	Yes
	15	21		- - -	Yes
	20	30		- - -	Yes
	25	36		- - -	Yes
	50	72		- - -	Yes
Bound pyruvic acid	5		3.5	- - -	Yes
	10		7	- - -	Yes
	15		10	- - -	Yes
	20		15	- - -	Yes
	25		18	- - -	Yes
	50		36	- - -	Yes
Bound acetaldehyde, pyruvic acid	5	8	3.5	- - -	Yes
	10	14.5	7	- - -	Yes
	15	21	10	- - -	Yes
	20	30	15	- - -	Yes
	25	36	18	- - -	Yes
	50	72	36	- - -	Yes

- - - Very delayed growth as compared to control; No growth in 28days
- - Delayed growth as compared to control; 10 days ≤ Growth ≤ 28 days
- Slightly delayed growth as compared to control; Growth ≤ 10 days
+/- Growth same as control
+ Slight stimulation of growth as compared to the control
+ + Stimulated growth as compared to the control
+ + + Strongly stimulated growth as compared to control; growth > control

Table 4.7: Summary of the growth response of *P. damnosus* in Hood media at pH 3.7 containing either SO_2, acetaldehyde, pyruvic acid, acetaldehyde bound SO_2, pyruvic acid bound SO_2, or acetaldehyde and pyruvic acid bound SO_2 and whether or not degradation of acetaldehyde or pyruvic acid occurred.

Treatment	SO_2 (mg/L)	Acetaldehyde (mg/L)	Pyruvic acid (mg/L)	Growth response	Degradation
SO_2	5			-	n/a
	10			-	n/a
	15			-	n/a
	20			-	n/a
	25			-	n/a
	50			- -	n/a
Acetaldehyde		8		+ + +	Yes
		14.5		+ + +	Yes
		21		+ + +	Yes
		30		+ + +	Yes
		36		+ + +	Yes
		72		+ + +	Yes
Pyruvic acid			3.5	- -	Yes
			7	- -	Yes
			10	- -	Yes
			15	- -	Yes
			18	- -	Yes
			36	- -	Yes
Bound acetaldehyde	5	8		- -	Yes
	10	14.5		- -	Yes
	15	21		- -	Yes
	20	30		- -	Yes
	25	36		- -	Yes
	50	72		- -	Yes
Bound pyruvic acid	5		3.5	-	Yes
	10		7	-	Yes
	15		10	-	Yes
	20		15	-	Yes
	25		18	-	Yes
	50		36	-	Yes
Bound acetaldehyde, pyruvic acid	5	8	3.5	- -	Yes
	10	14.5	7	- -	Yes
	15	21	10	- -	Yes
	20	30	15	- -	Yes
	25	36	18	- -	Yes
	50	72	36	- -	Yes

- - - Very delayed growth as compared to control; No growth in 28days
- - Delayed growth as compared to control; 10 days ≤ Growth ≤ 28 days
- Slightly delayed growth as compared to control; Growth ≤ 10 days
+/- Growth same as control
+ Slight stimulation of growth as compared to the control
+ + Stimulated growth as compared to the control
+ + + Strongly stimulated growth as compared to control; growth > control

Table 4.8: Summary of the growth response of *Lb. hilgardii* in Hood media at pH 3.5 containing either SO_2, acetaldehyde, pyruvic acid, acetaldehyde bound SO_2, pyruvic acid bound SO_2, or acetaldehyde and pyruvic acid bound SO_2 and whether or not degradation of acetaldehyde or pyruvic acid occurred.

Treatment	SO_2 (mg/L)	Acetaldehyde (mg/L)	Pyruvic acid (mg/L)	Growth response	Degradation
SO_2	5			- - -	n/a
	10			- - -	n/a
	15			- - -	n/a
	20			- - -	n/a
	25			- - -	n/a
	50			- - -	n/a
Acetaldehyde		8		+ +	Yes
		14.5		+ +	Yes
		21		+ +	Yes
		30		+ +	Yes
		36		+ +	Yes
		72		+ +	Yes
Pyruvic acid			3.5	+ / -	Yes
			7	+ / -	Yes
			10	+ / -	Yes
			15	+ / -	Yes
			18	+ / -	Yes
			36	+ + +	Yes
Bound acetaldehyde	5	8		- -	Yes
	10	14.5		- -	Yes
	15	21		- -	Yes
	20	30		- -	Yes
	25	36		- -	Yes
	50	72		- -	Yes
Bound pyruvic acid	5		3.5	- - -	Yes
	10		7	- - -	Yes
	15		10	- - -	Yes
	20		15	- - -	Yes
	25		18	- - -	Yes
	50		36	- - -	Yes
Bound acetaldehyde, pyruvic acid	5	8	3.5	- - -	Yes
	10	14.5	7	- - -	Yes
	15	21	10	- - -	Yes
	20	30	15	- - -	Yes
	25	36	18	- - -	Yes
	50	72	36	- - -	Yes

- - - Very delayed growth as compared to control; No growth in 28days
- - Delayed growth as compared to control; 10 days ≤ Growth ≤ 28 days
- Slightly delayed growth as compared to control; Growth ≤ 10 days
+/- Growth same as control
+ Slight stimulation of growth as compared to the control
+ + Stimulated growth as compared to the control
+ + + Strongly stimulated growth as compared to control; growth > control

Table 4.9: Summary of the growth response of *Lb. hilgardii* in Hood media at pH 3.7 containing either SO_2, acetaldehyde, pyruvic acid, acetaldehyde bound SO_2, pyruvic acid bound SO_2, or acetaldehyde and pyruvic acid bound SO_2 and whether or not degradation of acetaldehyde or pyruvic acid occurred.

Treatment	SO_2 (mg/L)	Acetaldehyde (mg/L)	Pyruvic acid (mg/L)	Growth response	Degradation
SO_2	5			- - -	n/a
	10			- - -	n/a
	15			- - -	n/a
	20			- - -	n/a
	25			- - -	n/a
	50			- - -	n/a
Acetaldehyde		8		+ / -	Yes
		14.5		+ / -	Yes
		21		+ / -	Yes
		30		+ / -	Yes
		36		+ / -	Yes
		72		+ / -	Yes
Pyruvic acid			3.5	+ / -	Yes
			7	+ / -	Yes
			10	+ / -	Yes
			15	+ / -	Yes
			18	+ / -	Yes
			36	+ / -	Yes
Bound acetaldehyde	5	8		- - -	Yes
	10	14.5		- - -	Yes
	15	21		- - -	Yes
	20	30		- - -	Yes
	25	36		- - -	Yes
	50	72		- - -	Yes
Bound pyruvic acid	5		3.5	+/-	Yes
	10		7	- -	Yes
	15		10	-	Yes
	20		15	- - -	Yes
	25		18	- - -	Yes
	50		36	- - -	Yes
Bound acetaldehyde, pyruvic acid	5	8	3.5	-	Yes
	10	14.5	7	- -	Yes
	15	21	10	- - -	Yes
	20	30	15	- -	Yes
	25	36	18	- - -	Yes
	50	72	36	- - -	Yes

- - - Very delayed growth as compared to control; No growth in 28days
- - Delayed growth as compared to control; 10 days ≤ Growth ≤ 28 days
- Slightly delayed growth as compared to control; Growth ≤ 10 days
+/- Growth same as control
+ Slight stimulation of growth as compared to the control
+ + Stimulated growth as compared to the control
+ + + Strongly stimulated growth as compared to control; growth > control

DISCUSSION

During this study several LAB species responded differently during growth in media containing free and bound SO_2. *O. oeni* VFO, *Lb. hilgardii*, and *P. parvulus* appeared very sensitive to both free and bound SO_2 at relatively low concentrations while *P. damnosus* was more resistant. Although there is some conflict in the literature regarding the relative sensitivity of wine LAB species to SO_2, in general it is reported that species of *Lactobacillus* and *Pediococcus* are more tolerant of SO_2 than *O. oeni* (Davis *et al*., 1988; Hood, 1983; Carr *et al*., 1976; Lafon-Lafourcade and Peynaud, 1974; Fornachon, 1963), findings supported by this current study. In addition, this study also demonstrated that *P. damnosus* was more tolerant to SO_2 than *P. parvulus*, a finding not previously reported.

The concentrations of SO_2 that were inhibitory to the LAB during this study were generally quite low compared to inhibitory concentrations previously reported. For example, Davis *et al*. (1988) reported that all strains of *O. oeni*, *P. parvulus,* and *Lactobacillus* spp. tested grew in the presence of up to 64 mg/L of total SO_2. Lafon-Lafourcade *et al*. (1983) also showed similar growth of LAB when 50 mg/L total SO_2 was added to the medium. In contrast, Carr *et al*. (1974) saw inhibition of *Lactobacillus* spp. and *O. oeni* at 5 mg/L total SO_2. However, when removed from the medium containing SO_2 6 strains grew, suggesting that the majority were not killed but suppressed by SO_2. Some of this variation may be explained by the use of different growth media in these studies. In this study a minimal media was utilized that contained very low concentrations of any compounds that could bind SO_2. In addition, the media contained minimal concentrations of carbohydrates, nitrogen, vitamins, and minerals, making

growth difficult for the fastidious LAB. It also resembles wine more closely than other artificial media used in previous work (Osborne *et al*., 2000; Davis *et al,* 1988; Carr *et al*., 1976; Lafon-Lafourcade and Peynaud, 1974; Fornachon, 1963) particularly in having low levels of sugars, similar to what is observed post alcoholic fermentation when LAB are most commonly to grow (Osborne and Edwards, 2006; Alexandre *et al*., 2004; Wibowo *et al*., 1985). Finally, Lafon-Lafoucade and Peynaud (1974) also noted that free SO_2 was more effective in simple media, as compared to wine media, and attributed this to the lower binding capacities of the simple media than in the more complex media.

Aside from media differences, many previous reports on the effect of SO_2 on wine LAB utilized growth media at pH values higher than typical wine pH (3 to 4). This makes it difficult to compare results from growth studies particularly when SO_2 is involved as this compound is known to be much more effective at low pH (Strantford *et al*., 1987; Macris and Markakis, 1974). For example, Fornachon (1963) used a diluted grape juice with a final pH of 4.2 while Carr *et al*. (1976) used media where the pH was adjusted to 4.0. Although growth in the free SO_2 treatments was minimal it is important to note that these results reflect the interaction of pH and SO_2 rather than the effects of pH alone since all bacterial controls grew at the lowest pH in the absence of SO_2.

Although inhibition of wine LAB is known to be caused by free SO_2, and in particular molecular SO_2 (Rose and Pilkington, 1989; Hinze and Holzer, 1986; Eschenbruch, 1974; Hammond and Carr, 1976), there is still some debate regarding the inhibitory action of bound SO_2 (Osborne and Edwards, 2006; Larsen *et al,* 2003; Hood, 1983). In this study, inhibition of bacterial growth was observed in media containing only bound SO_2, as confirmed by aeration-oxidation, supporting the findings of other

researchers that bound SO_2 is more inhibitory then previously reported (Osborne and Edwards, 2006; Larsen *et al,* 2003; Hood, 1983; Fornachon, 1963).

Of the three types of bound SO_2 used in this experiment, the effects of acetaldehyde bound SO_2 on the growth of all four LAB was the most significant in inhibiting growth. However, the effects of pyruvic acid bound SO_2 and the combination of acetaldehyde and pyruvic acid bound SO_2 displayed similar inhibitory effects at both pH 3.5 and 3.7. Acetaldehyde bound SO_2 at pH 3.5 was the most inhibitory to LAB growth with the lower pH strongly enhancing the antibacterial activity of the acetaldehyde bound SO_2, supporting work by Osborne *et al.* (2006), Hood (1983), and Lafon-Lafourcade and Peynaud (1974). Free SO_2 is more inhibitory at lower pH values and so it is not surprising that bound SO_2 would also behave in the same way. *O. oeni* VFO was the most sensitive bacteria to acetaldehyde bound SO_2 at both pH 3.5 and 3.7 in contrast to what was reported by Hood (1983) where Pediococci were found to be the most sensitive bacteria tested to acetaldehyde bound SO_2. In fact, in this present study *P. damnosus* was observed to be least effected by acetaldehyde bound SO_2 and showed growth at pH 3.5 and 3.7. It should be noted that Hood (1983) tested strains of *P. cerevisiae* and *P. pentacaceous* in contrast to *P. damnosus* and *P. parvulus* used in this present study. In light of this it, there seems to be some variation between *Pediococcus* species with regards to SO_2 tolerance.

Compared to acetaldehyde bound SO_2, pyruvic acid bound SO_2 and the combination of acetaldehyde and pyruvic acid bound SO_2 was less inhibitory to the LAB with some growth being observed at pH 3.7. This was surprising given that low levels of free SO_2 were always present in the pyruvic acid bound SO_2 treatments due to the lower

affinity of pyruvic acid for SO_2. These results are contrary to what was observed by Lafon-Lourcade and Peynaud (1974) who suggested that pyruvic acid bound SO_2 was more inhibitory than acetaldehyde bound SO_2. However, little detail is given regarding the conditions of this study including the concentrations of free SO_2 and pyruvic acid bound SO_2. Reasons for this are unclear since the amount of measured free SO_2 in both the SO_2 and pyruvic acid bound SO_2 treatments were relatively the same and yet complete growth inhibition was observed in one case but not the other. It is possible that the presence of free pyruvic acid in the pyruvic acid bound SO_2 treatments may have minimized the inhibitory impact of the bound SO_2. In support of this, stimulation of growth in media containing pyruvic acid was observed in this study.

Stimulation of growth in media containing acetaldehyde was observed for *O. oeni* VFO and *Lb. hilgardii* but delayed growth by *P. damnosus* and *P. parvulus* at pH 3.5. At pH 3.7 all strains displayed growth similar to the control suggesting that acetaldehyde metabolism provided an advantage at lower pH when bacteria were under more stress but not at higher pH. Metabolism of acetaldehyde by wine LAB has been reported before with Osborne *et al.* (2000) suggesting a mechanism for stimulated energy production in which acetaldehyde may be reduced to ethanol allowing recycling of NAD^+. This recycling of NAD^+ means that acetyl-phosphate is now available to be converted to acetic acid through the action of the enzyme acetate kinase. This is an ATP generating process and so would be energetically advantageous for the bacteria. Although Osborne *et al.,* (2000) demonstrated degradation of acetaldehyde by wine LAB, stimulation of growth has not to date been demonstrated. In addition, in this study *P. damnosus* and *P. parvulus* was shown to degrade acetaldehyde, a finding in contrast to what was reported by

Osborne *et al*. (2000) where two *Pediococcus* strains tested did not degrade acetaldehyde. It should be noted though, that the study by Osborne *et al.* (2000) was performed with resting cells in a buffered system in contrast to this present study where studies were performed in a media similar to wine.

In media containing pyruvic acid, *O. oeni* VFO exhibited stimulated growth at pH 3.5 but not pH 3.7 while growth similar to the control was observed by strains *P. parvulus*, *P. damnosus,* and *Lb. hilgardii* at both pH values. To date stimulated growth of wine LAB by pyruvic acid has not been documented and the mechanism by which this stimulation occurs is unknown. However, given the central role pyruvic acid plays in bacterial metabolism stimulation of growth in the presence of pyruvic acid is not unusual.

While all LAB tested could degrade both acetaldehyde and pyruvic acid, they were also able to degrade SO_2 bound acetaldehyde. Furthermore, apart from *O. oeni* VFO, degradation of SO_2 bound pyruvic acid was also observed. While the ability of wine LAB to degrade SO_2 bound acetaldehyde has previously been reported (Osborne *et al.,* 2006), this is the first report of wine LAB being able to degrade SO_2 bound pyruvic acid. However, degradation of either SO_2 bound acetaldehyde or SO_2 bound pyruvic acid did not correlate with growth of the bacteria. In fact, inhibition of bacterial growth was always observed in media containing bound SO_2 even though a measured reduction in either acetaldehyde or pyruvic acid was observed. This observation lends some weight to an explanation of how bound SO_2 may inhibit bacterial growth. A number of researchers have proposed that the inhibitory effect of bound SO_2 is due to the release of free SO_2 when the bound acetaldehyde, and/or in this case, pyruvic acid is metabolized by the bacteria. (Larsen *et al,* 2003; Osborne *et al*., 2000; Hood, 1983; Fornachon, 1963). This is

in contrast to Mayer *et al*. (1976) who theorized that inhibition was the result of the bound SO_2 complex itself and not the release of free SO_2. In this current study, metabolic activity (degradation of acetaldehyde and pyruvic acid) was observed after inoculation suggesting that the bacteria were not immediately inhibited by the bound SO_2. However, after some degradation of acetaldehyde and/or pyruvic acid had occurred this metabolic activity ceased. Because no growth was observed by an increase in OD, the degradation of acetaldehyde and pyruvic acid may have been caused by resting cells of the bacteria rather than actively growing cells. Alternatively, growth may have occurred but the magnitude of it was not large enough to cause an observable increase in OD.

LAB inhibition by the release of SO_2 from the bound form could possibly be the result of bacteriostatic action by the intracellular accumulation of the bisulfite (Hood, 1983; Mayer *et al*., 1975; Lafon-Lourcade and Peynaud, 1974) as is the case for inhibition by molecular SO_2. This may have occurred due to bound SO_2 crossing the cell membrane and subsequent dissociation of the acetaldehyde or pyruvic acid moiety (due to the change in pH). However there are discrepancies in the literature as to whether bound SO_2 could cross the bacterial cell membrane. In some cases the bound SO_2 complex is reported as being charged (Azevedo *et al*., 2007; Fugelsang and Edwards, 2007; Dufour *et al*., 1999) while in others it is reported as being uncharged (Burroughs and Sparks, 1973). In both cases it is difficult to ascertain whether the authors were describing the charge of bound SO_2 at typical wine pHs. Yet the reduction of acetaldehyde and pyruvic acid give some evidence that bound SO_2 is able to pass the through the cell membrane since no extracellular enzymes capable of metabolizing acetaldehyde or pyruvic acid have been found in wine LAB. It is also known that

stressful conditions, such as growth in the presence of SO_2, ethanol, and low pH, can affect the permeability of the cell membrane of *O. oeni* (Garbay and Lonvaud-Funel, 1996; Garbay and Lonvaud-Funel, 1994) and disrupt ATPase activity (Carrete *et al.,* 2002). This may impact the ability of bound SO_2 to pass through the cell membrane and requires more study.

CONCLUSION

Growth studies demonstrated strains of *O. oeni* VFO, *P. parvulus,* and *Lb. hilgardii* were inhibited in media containing free SO_2, acetaldehyde bound SO_2, pyruvic acid bound SO_2, at both pH 3.5 and 3.7. *P. damnosus* was the most tolerant to both free and bound SO_2 at both pH 3.5 and 3.7. In general, acetaldehyde bound SO_2 was more inhibitory than pyruvic acid bound SO_2 or the acetaldehyde and pyruvic acid bound SO_2 combination. Degradation of SO_2 bound acetaldehyde was observed for *O. oeni* VFO, *P. parvulus, P. damnosus,* and *Lb. hilgardii* and degradation of SO_2 bound pyruvic acid was observed for *P. parvulus, P. damnosus,* and *Lb. hilgardii*. In contrast, stimulation of growth in media containing acetaldehyde was observed for *O. oeni* VFO and *Lb. hilgardii* but not *P. parvulus* or *P. damnosus* at pH 3.5. At pH 3.7 all LAB demonstrated growth similar to the control. In media containing pyruvic acid *O. oeni* VFO exhibited stimulated growth at pH 3.5 but not pH 3.7 while growth similar to the control was observed by strains *P. parvulus*, *P. damnosus,* and *Lb. hilgardii* at both pH values.

Results from this study provide additional information regarding the impact of SO_2 and bound SO_2 on growth of wine LAB. Bound SO_2 is an important component in wine and little is known about its effect on wine LAB. Future work should focus on the

investigation of a larger number of wine LAB species to investigate if the trends observed in this study are valid. In particular, *Pediococcus* species should be investigated as they demonstrated varied tolerance to SO_2 and are a problematic spoilage organism in wine. Future research should also include investigating the mechanism by which bound SO_2 is causing bacterial inhibition and in particular, if and how bound SO_2 crosses the cell membrane.

CHAPTER 5
General Conclusions and Summary

During growth in both a synthetic grape juice and Pinot gris juice various strains of *S. cerevisiae* produced significantly different concentrations of SO_2, acetaldehyde, and pyruvic acid but not α-ketoglutaric acid. All of these compounds were produced in the highest amounts during early to mid fermentation during yeast exponential growth. Yeast strains that produced the highest levels of SO_2 were strongly inhibitory to the MLF. Because little to no free SO_2 was measured during the fermentations, inhibition may have been due to bound SO_2. In this study it appears that bound SO_2 was predominately acetaldehyde bound SO_2 due to the high concentrations of acetaldehyde produced by the yeast strains. Conversely, results also showed that inhibition of MLF was not always correlated to SO_2 production indicating other mechanisms of inhibition.

To further elucidate the role of bound SO_2 in the inhibition of wine LAB, growth studies were conducted where various wine LAB were grown in media containing free SO_2 or acetaldehyde and pyruvic acid bound SO_2 at two different pHs. Inhibition was greater in media at pH 3.5 than at 3.7 with most wine LAB not showing signs of growth in media containing only free SO_2. *P. damnosus* was the most tolerant to both free and bound SO_2 at both pH 3.5 and 3.7. In general, acetaldehyde bound SO_2 was more inhibitory than pyruvic acid bound SO_2 or the acetaldehyde and pyruvic acid bound SO_2 combination. A reduction of SO_2 bound acetaldehyde was observed for *O. oeni* VFO, *P. parvulus, P. damnosus,* and *Lb. hilgardii.* A reduction of SO_2 bound pyruvic acid was observed for *P. parvulus, P. damnosus,* and *Lb. hilgardii*. In contrast, stimulation of growth in media containing acetaldehyde was observed for *O. oeni* VFO and *Lb.*

hilgardii but not *P. parvulus* or *P. damnosus* at pH 3.5. At pH 3.7 all LAB demonstrated growth similar to the control. In media containing pyruvic acid *O. oeni* VFO displayed stimulated growth at pH 3.5 but not pH 3.7 while growth similar to the control was observed by strains *P. parvulus*, *P. damnosus,* and *Lb. hilgardii* at both pH values.

Bound SO_2 is obviously an important component in wine and little is known about its effect on wine LAB. Future work should involve using a wider range of *O. oeni* strains to determine if there are any strain differences related to tolerance to bound SO_2. In addition, the impact of bound SO_2 on other wine spoilage bacteria should be studied. In particular, *Pediococcus* species should be investigated as they demonstrated varied tolerance to SO_2 and are a problematic spoilage organism in wine. Future research should also include investigating the mechanism by which bound SO_2 is causing bacterial inhibition, in particular, how bound SO_2 crosses the cell membrane.

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APPENDICES

APPENDIX A

A: Synthetic grape juice medium described by Wang *et al*. (2003), modified as per Osborne and Edwards (2006).

pH=3.5
Higher Sugars = 24%

Vitamins	**mg/L**
Myo-Inositol	100.00
Pyridoxine.HCl	2.00
Nicotinic acid	2.00
Calcium Pantothenate	0.25
Thiamin HCl	0.50
p-amino Benzoic Acid	0.20
Riboflavin	0.20
Folic acid	0.20
Biotin	10 ug/mL
Sugars and Salts	**g/L**
Glucose	120.00
Fructose	120.00
Potassium Tartrate	3.36
$CaCl_2 \bullet 2H_2O$	0.44
L- Malic acid	3.00
Citric acid H_2O	0.22
K_2HPO_4	1.14
$MgSO_4 \bullet 7H_2O$	1.23
Minerals	**mg/L**
$MnCl_2 \bullet 4H_2O$	0.20
$ZnCl_2$	0.14
$FeCl_2$	0.05
$CuCl_2$	0.01
H_3BO_3	0.01
$Co(NO_3)_2 \bullet 6\ H_2O$	0.03
$NaMoO_4 \bullet 2H_2O$	0.02
KIO_3	0.01
Purine & Pyrimidines	**mg/L**
Adenine sulphate	5.00
Guanine.HCl	5.00
Cytosine	5.00
Thymidine	5.00
Xanthine	5.00
Uracil	5.00
Other	
Tween 80 (5%)	1 mL/L

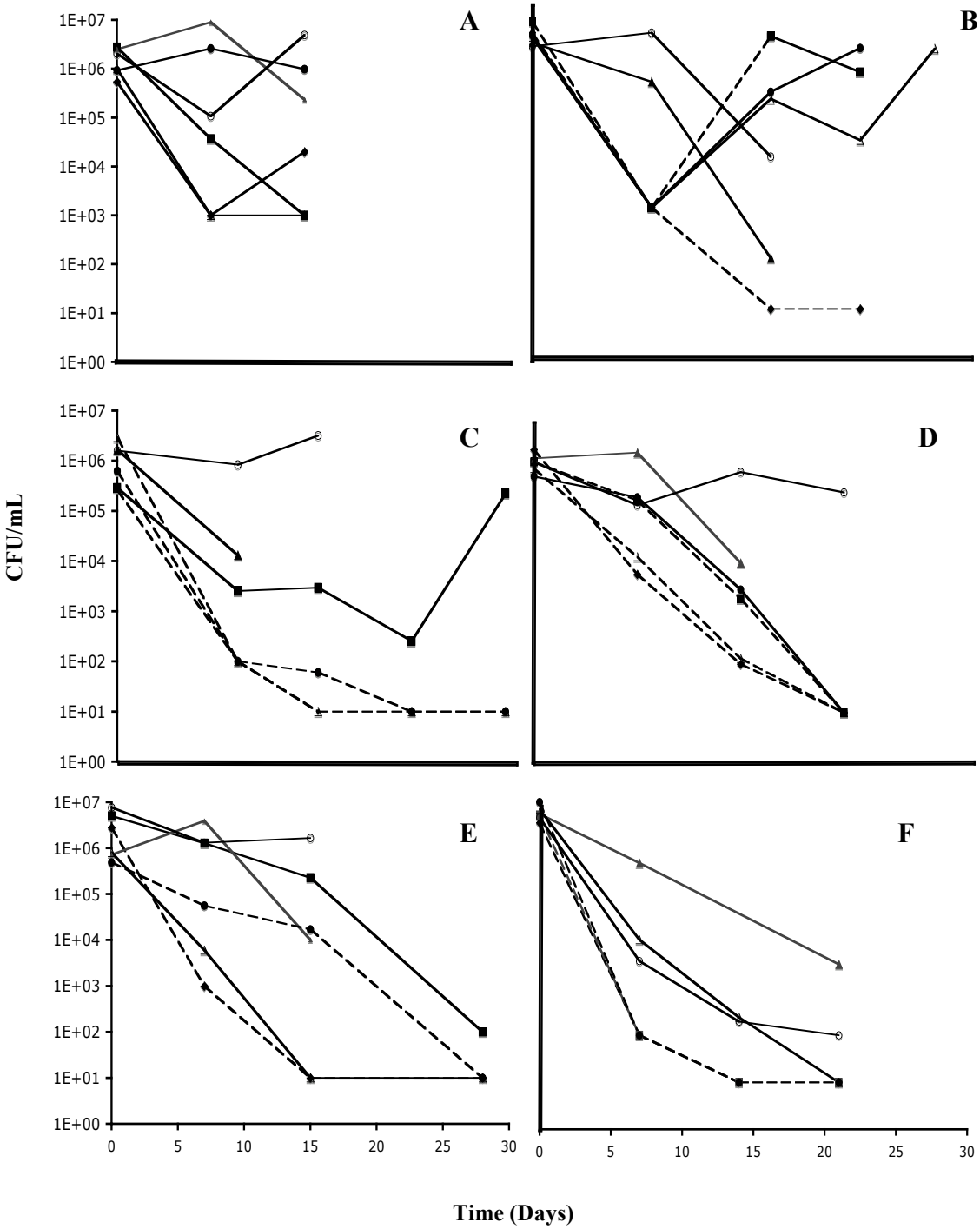
Synthetic grape juice (continued)

Amino Acids	**mg/L**
Alanine	161.00
Arginine	489.00
Aspartic acid	51.00
Cysteine	29.00
Glutamic acid	204.00
Glycine	15.00
Histidine	110.00
Isolueucine	66.00
Leucine	80.00
Lysine	95.00
Methionine	29.00
Phenlyalanine	66.00
Proline	5081.00
Serine	117.00
Threonine	102.00
Tryptophan	44.00
Tyrosine	51.00
Valine	445.00

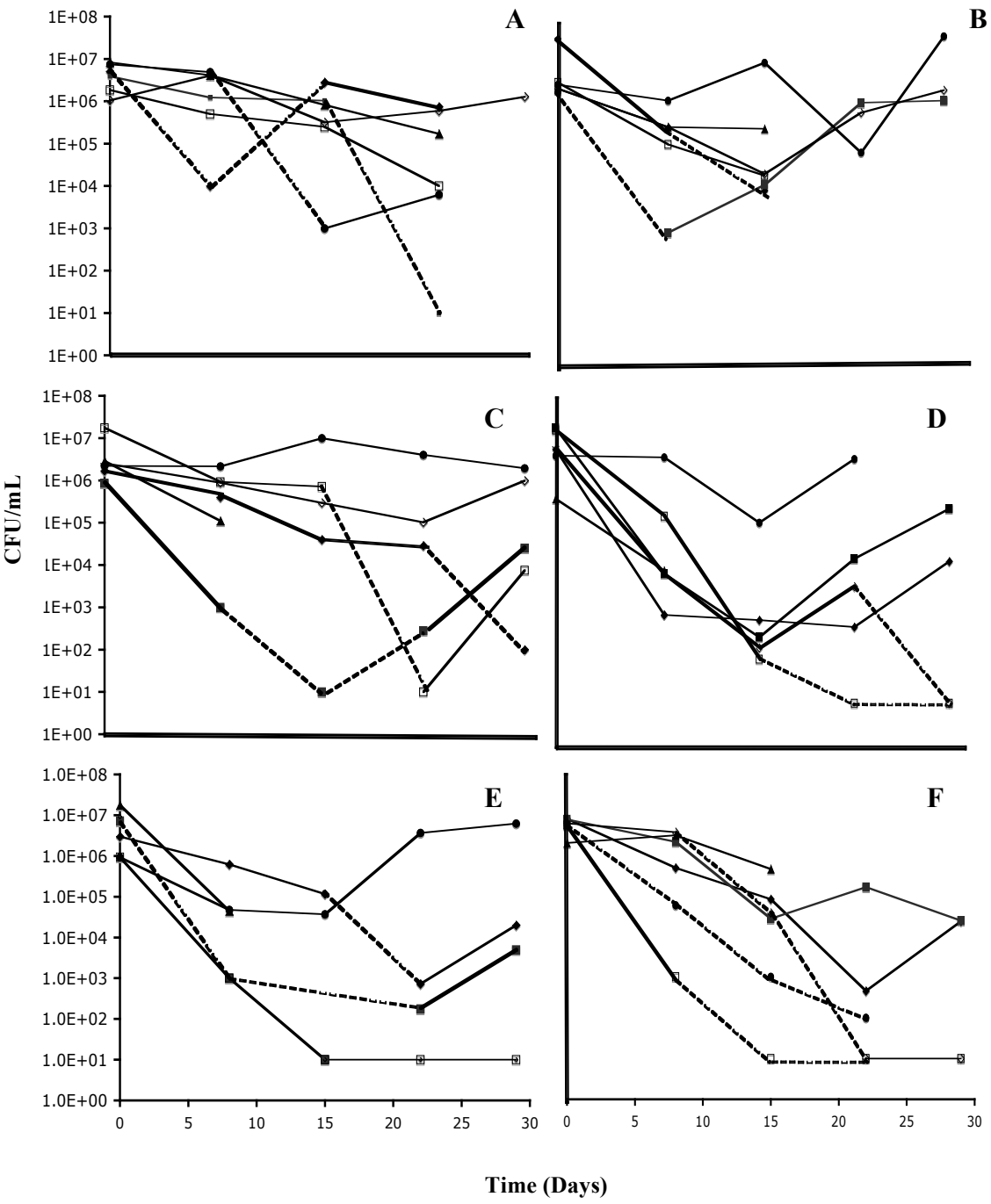
B: Residual Sugars after 29 or 50 days of the alcoholic fermentation for both the synthetic and Pinot gris juice[1] using the CliniTest® (g/100mL)[1].

Synthetic grape juice	**Day 29**
V1116	3.0
RUBY.ferm	3.7
MERIT.ferm	2.7
EC1118	1.0
S102	4.3
S325	4.3
M69	3.0
FX10	3.3
S6U	2.7
F15	2.3
BM45	2.0
43	2.6
Pinot gris juice	**Day 50**
V1116	3.3
FX10	5.0
BM45	3.0
M69	4.3

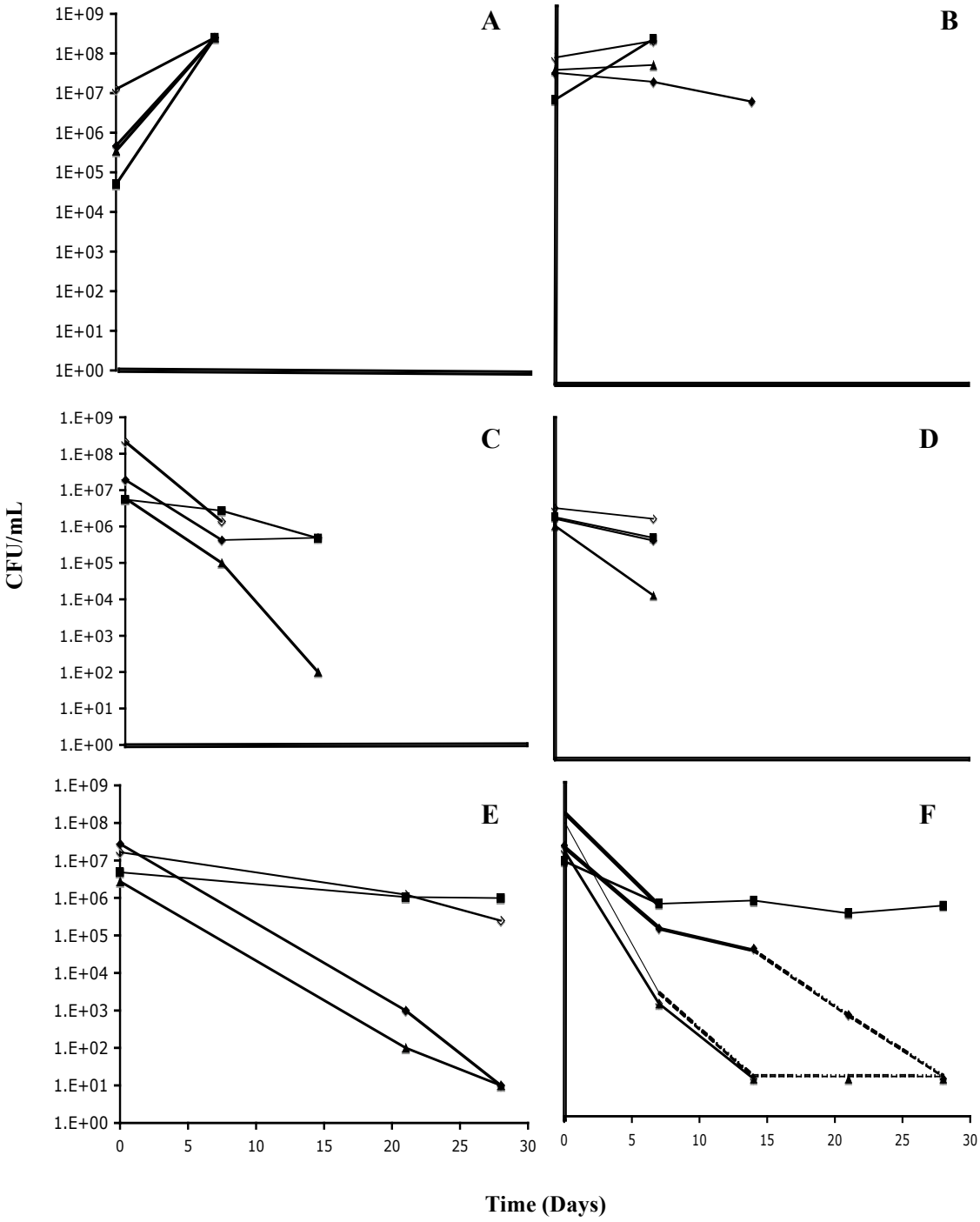
[1] Values are means of triplicate fermentations.



C: Growth of *O. oeni* VFO in synthetic grape juice undergoing alcoholic fermentation after 2 (A), 8 (B), 15 (C), 23 (D), 29 (E), and 50 (F) days induced by *S. cerevisiae* V1116 ◆; RUBY.ferm ■; MERIT.ferm ▲; S325 ●; EC1118 ○; S102 △. Values are means of triplicate fermentations.



D: Growth of *O. oeni* VFO in synthetic grape juice undergoing alcoholic after 2 (A), 8 (B), 15 (C), 23 (D), 29 (E), and 50 (F) days induced by *S. cerevisiae* M69 ◆; FX10 ■; S6U ▲; 43 ●; F15 ◇; BM45 □ . Values are means of triplicate fermentations.

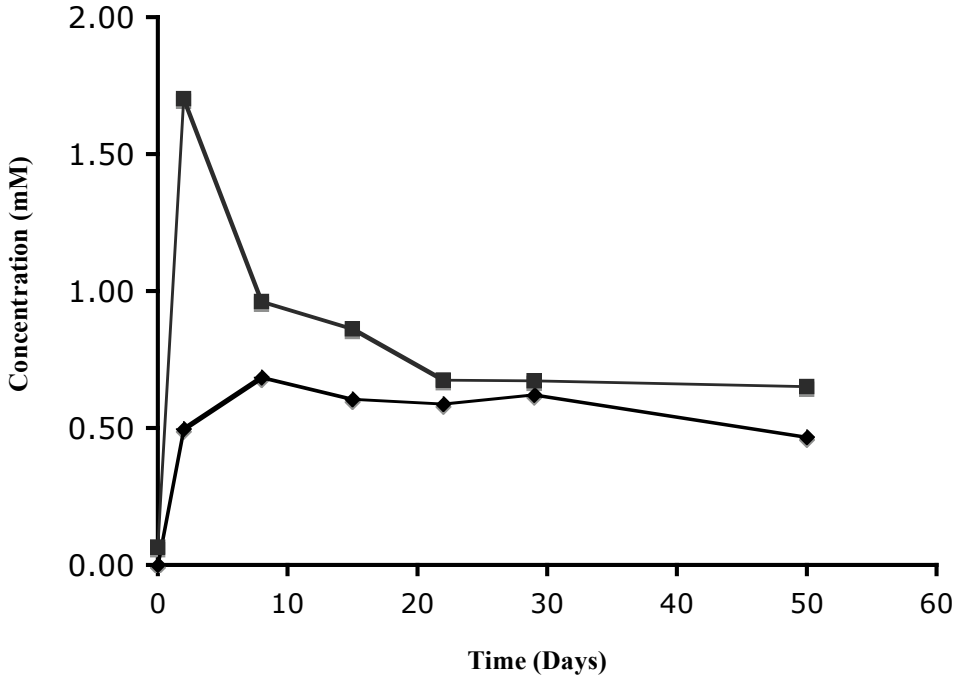


E: Growth of *O. oeni* VFO in Pinot gris grape juice undergoing alcoholic fermentation after 2 (A), 8 (B), 15 (C), 23 (D), 29 (E), and 50 (F) days induced by *S. cerevisiae* V1116 ◆; FX10 ■; BM45 ▲; M69 ◇. Values are means of triplicate fermentations.

F: Bound SO_2 and calculated binding compounds (mg/L) in Pinot gris juice during alcoholic fermentation for *S. cerevisiae* strain V1116.

Alcoholic Fermentation Day	Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	31.80	31.80	0.00	0.00
8	43.84	43.84	0.00	0.00
15	38.72	38.72	0.00	0.00
22	37.65	37.65	0.00	0.00
29	39.79	39.79	0.00	0.00
50	29.87	29.87	0.00	0.00

[1]Values are means of triplicate fermentations

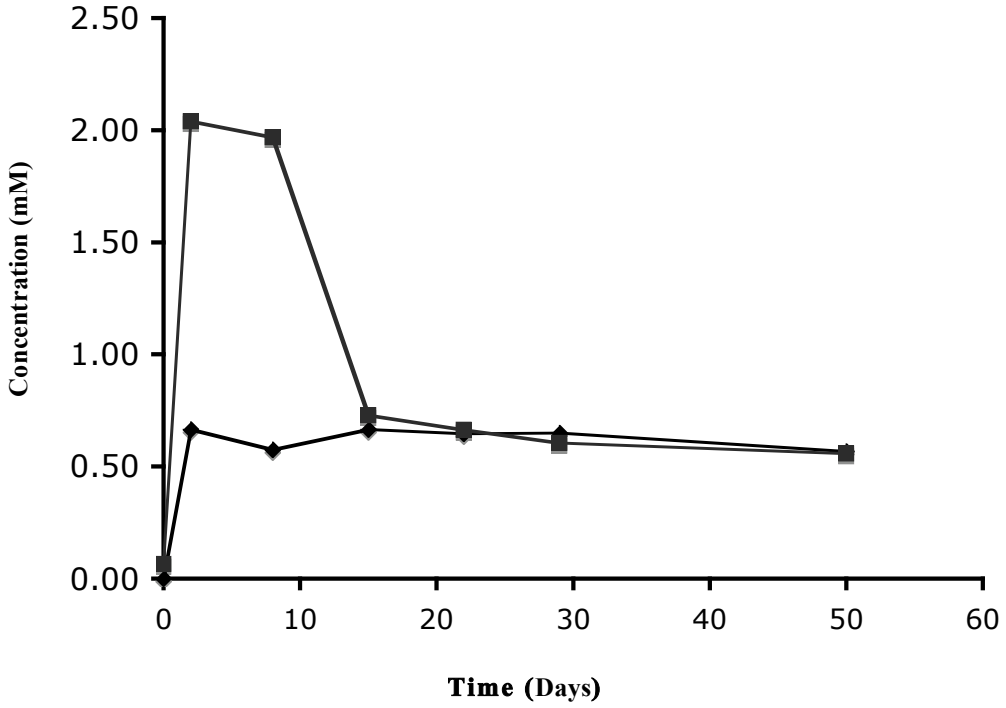


G: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain V1116 in Pinot gris juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

H: Bound SO_2 and calculated binding compounds (mg/L) in Pinot gris juice during alcoholic fermentation for *S. cerevisiae* strain BM45.

Alcoholic Fermentation Day	Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	42.56	42.56	0.00	0.00
8	36.80	36.80	0.00	0.00
15	42.56	44.02	1.97	1.08
22	41.39	41.21	2.11	1.98
29	41.60	34.86	1.12	0.32
50	36.27	34.69	1.87	1.07

[1]Values are means of triplicate fermentations

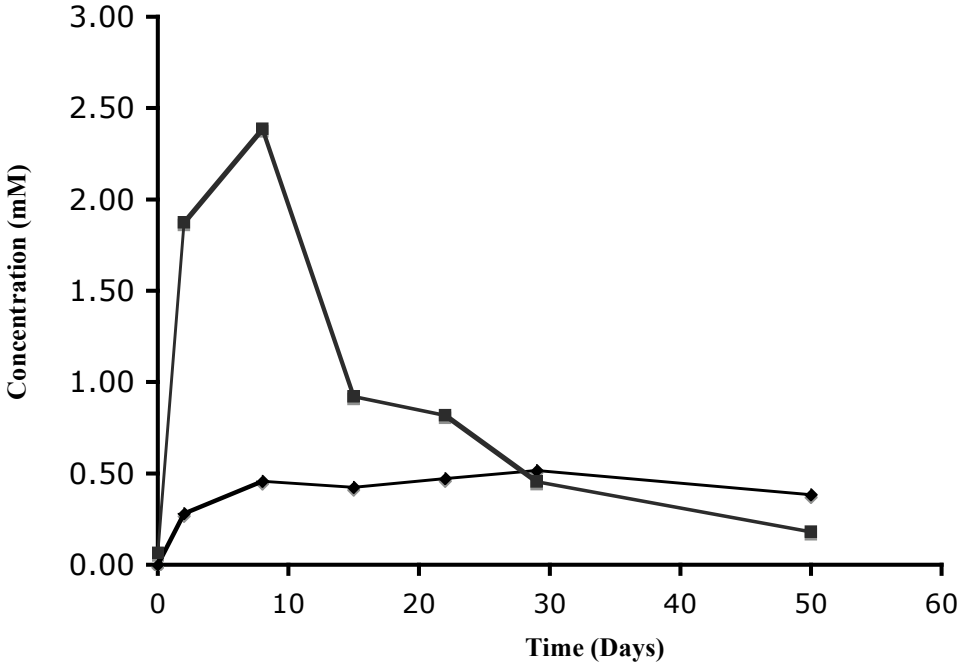


I: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain BM45 in Pinot gris juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

J: Bound SO_2 and calculated binding compounds (mg/L) in Pinot gris juice during alcoholic fermentation for *S. cerevisiae* strain M69.

Alcoholic Fermentation Day	Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	17.92	17.92	0.00	0.00
8	29.23	29.23	0.00	0.00
15	27.09	28.01	0.00	0.00
22	30.19	30.19	0.00	0.00
29	33.07	27.50	0.72	0.35
50	24.53	10.91	0.93	0.33

[1]Values are means of triplicate fermentations

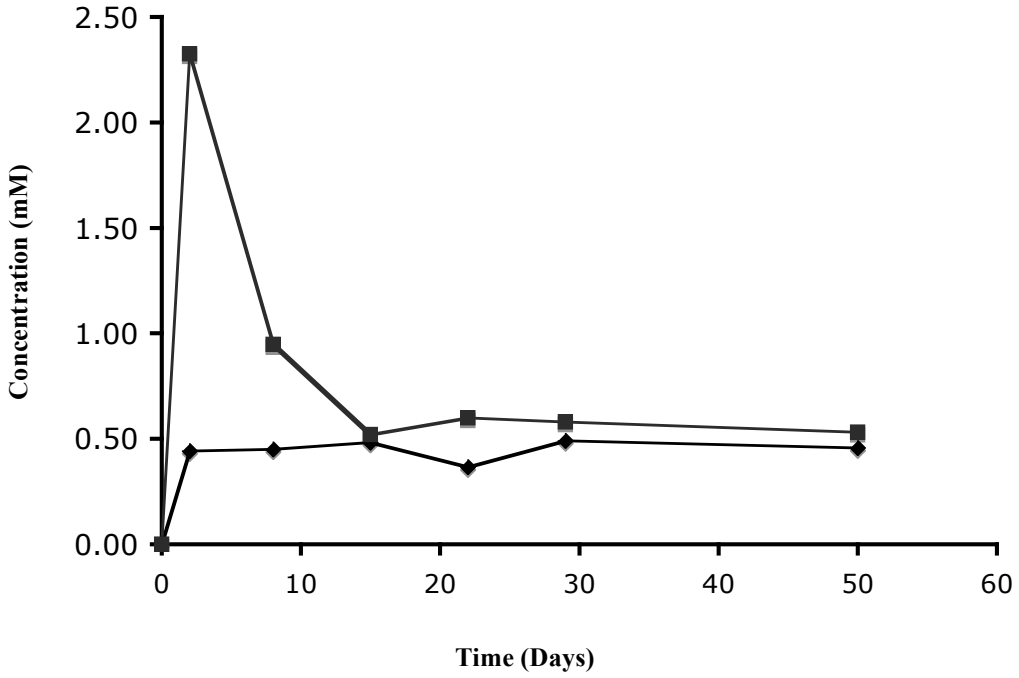


K: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain M69 in Pinot gris juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

L: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain V1116.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	28.27	0.00	0.00	0.00
8	28.80	28.80	0.00	0.00
15	30.93	31.84	0.00	0.72
22	23.47	23.47	0.00	0.57
29	31.47	34.08	0.00	0.47
50	29.23	31.25	0.00	0.45

[1]Values are means of triplicate fermentations

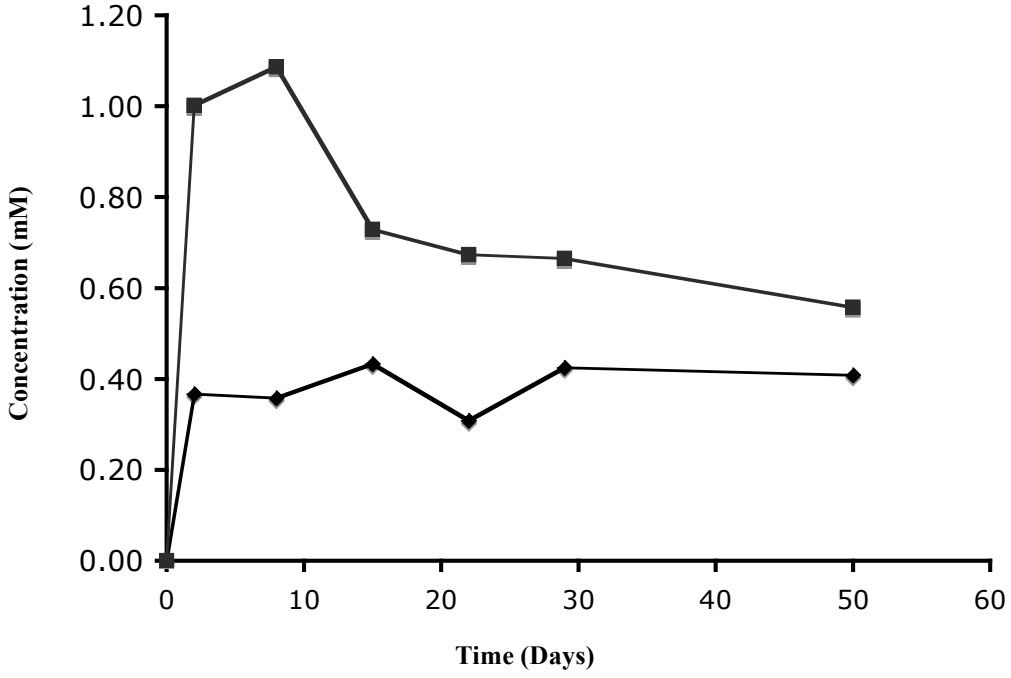


M: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain V1116 in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

N: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain RUBY.ferm.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	23.47	23.00	0.00	0.00
8	22.93	22.97	0.00	0.00
15	27.73	28.49	0.00	0.00
22	19.73	19.78	0.00	0.00
29	27.20	27.20	0.00	0.00
50	26.13	26.13	0.00	0.00

[1]Values are means of triplicate fermentations

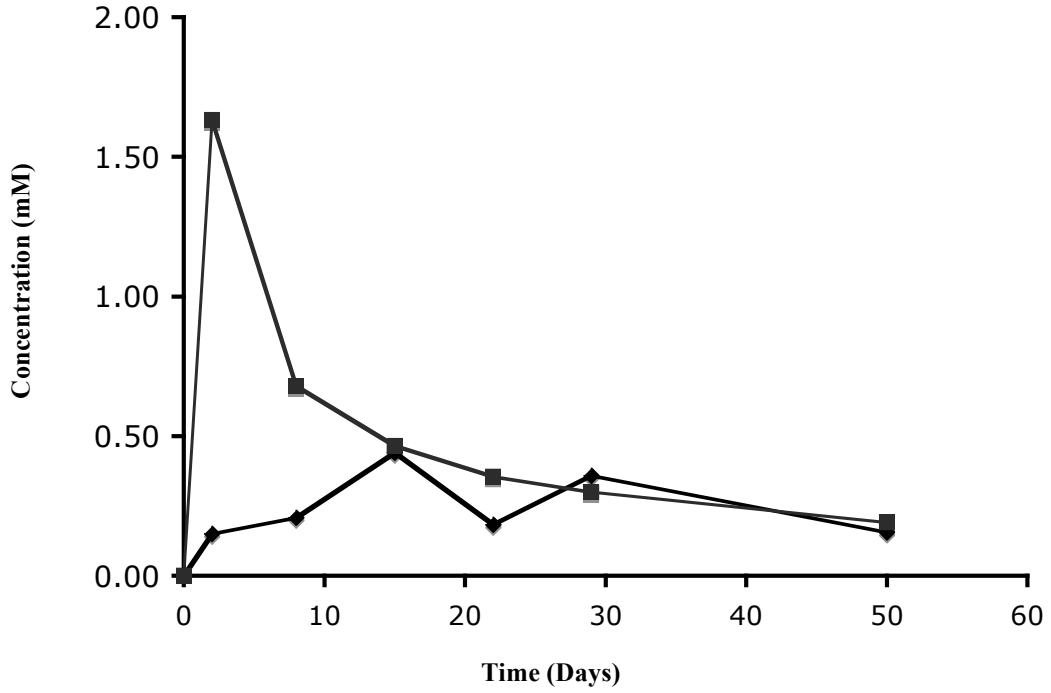


O: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain RUBY.ferm in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

P: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain MERIT.ferm.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0	0.00	0.00
2	9.60	9.86	0.00	0.00
8	13.33	16.86	0.00	0.00
15	28.27	25.24	3.75	0.19
22	11.73	12.56	0.00	0.00
29	22.93	17.74	6.57	0.34
50	9.92	10.65	0.76	0.79

[1]Values are means of triplicate fermentations

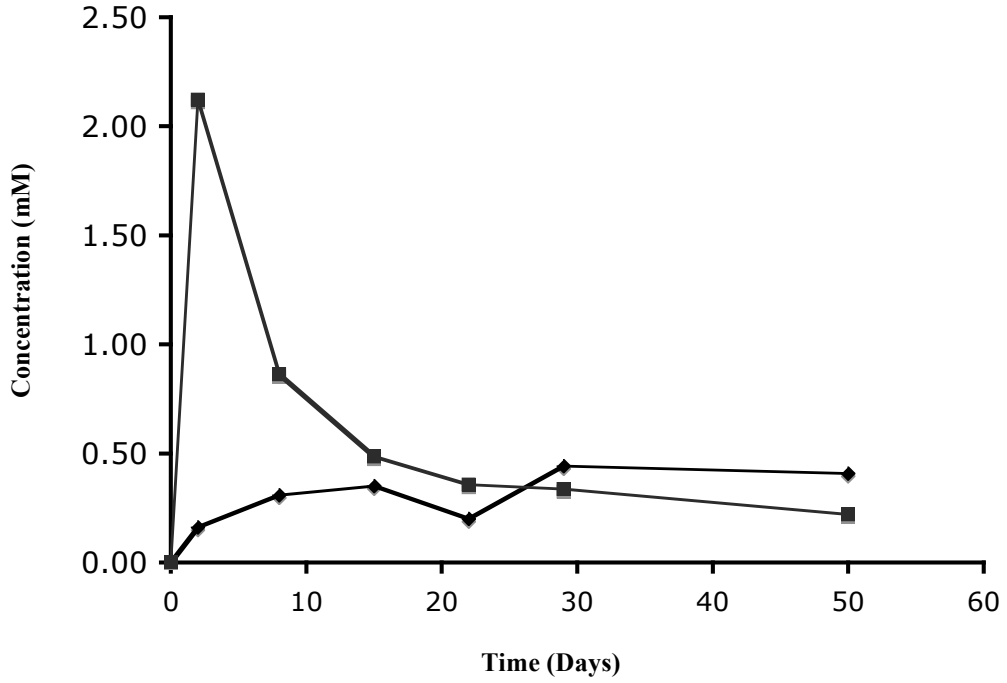


Q: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain MERIT.ferm in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

R: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain EC1118.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0	0.00	0.00
2	10.35	10.00	0.00	0.00
8	19.73	19.31	0.58	0.49
15	22.40	22.28	0.98	0.99
22	12.80	12.33	0.00	0.00
29	28.27	7.70	0.32	0.02
50	26.13	13.85	18.91	1.02

[1]Values are means of triplicate fermentations

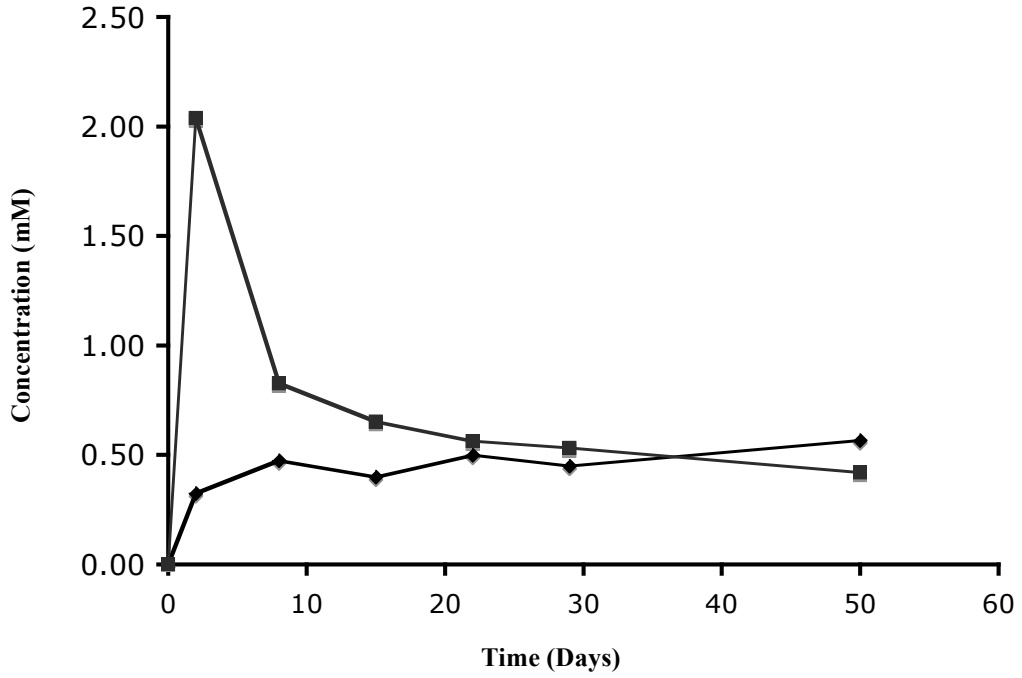


S: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain EC1118 in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

T: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain S102.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0	0	0.00	0.00
2	20.8	20.00	0.00	0.00
8	30.4	30.00	0.00	0.00
15	25.6	25.71	0.00	0.00
22	32	35.11	0.00	0.00
29	28.8	28.90	0.00	0.00
50	36.27	25.78	12.04	0.84

[1]Values are means of triplicate fermentations

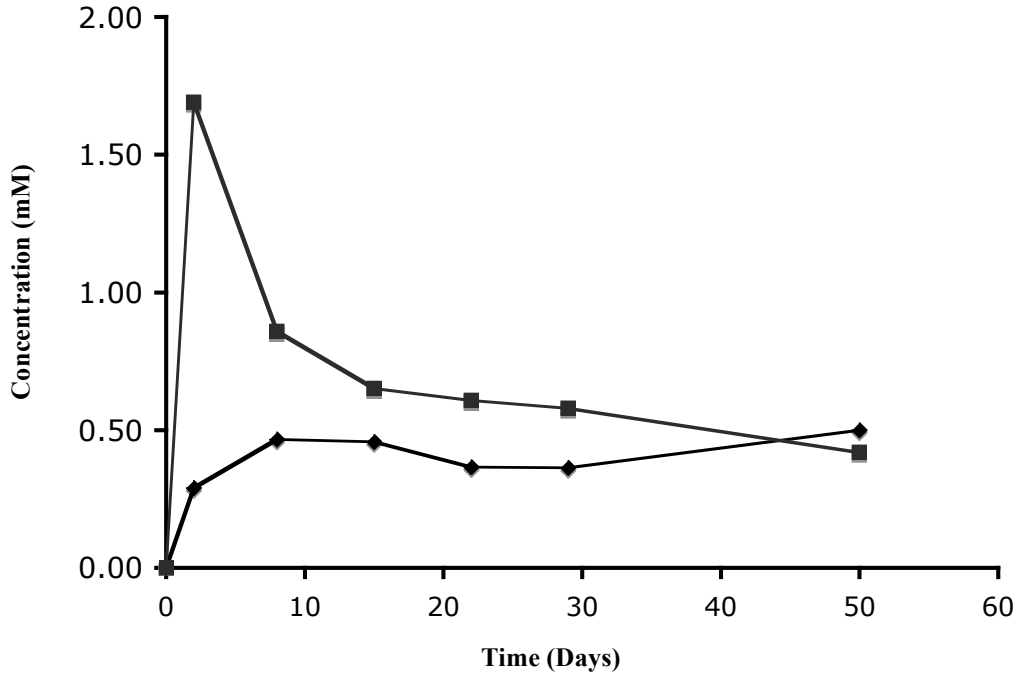


U: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain S102 in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

V: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain S325.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0	0.00	0.00
2	18.56	0.00	0.00	0.00
8	29.87	29.97	0.00	0.00
15	29.33	29.49	0.00	0.00
22	23.47	26.71	0.00	0.00
29	23.25	23.00	0.00	0.00
50	32.00	25.32	8.57	0.56

[1]Values are means of triplicate fermentations

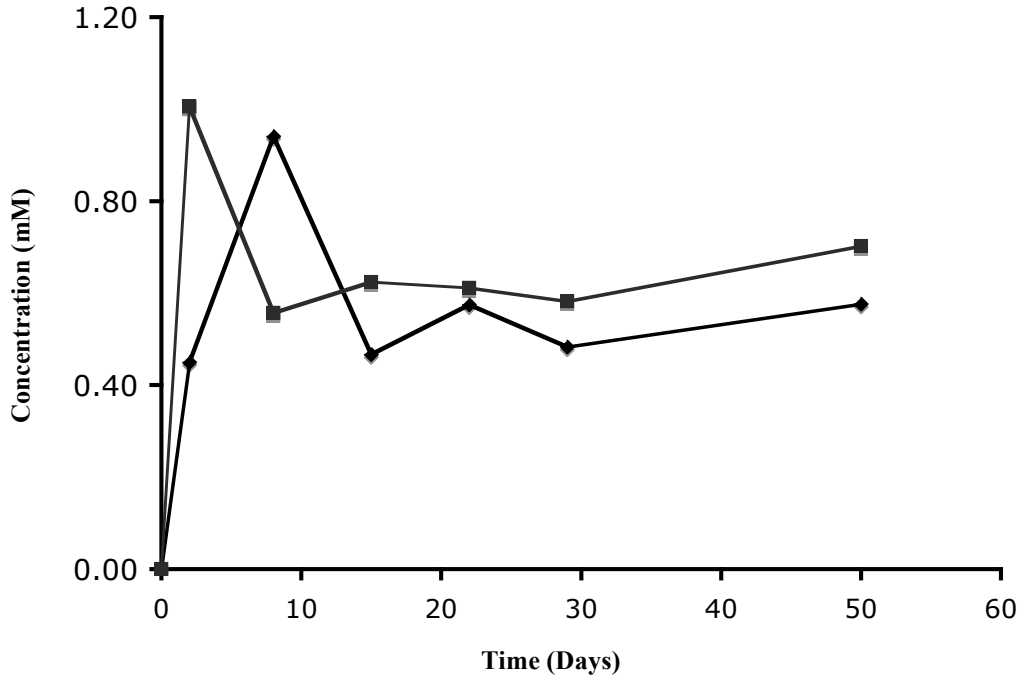


W: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain S325 in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

X: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain M69.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	28.80	28.55	0.00	0.00
8	60.27	34.11	11.12	0.94
15	29.87	29.37	0.00	0.00
22	36.80	38.42	0.00	0.00
29	30.93	30.33	0.00	0.00
50	36.91	36.04	0.00	0.00

[1]Values are means of triplicate fermentations

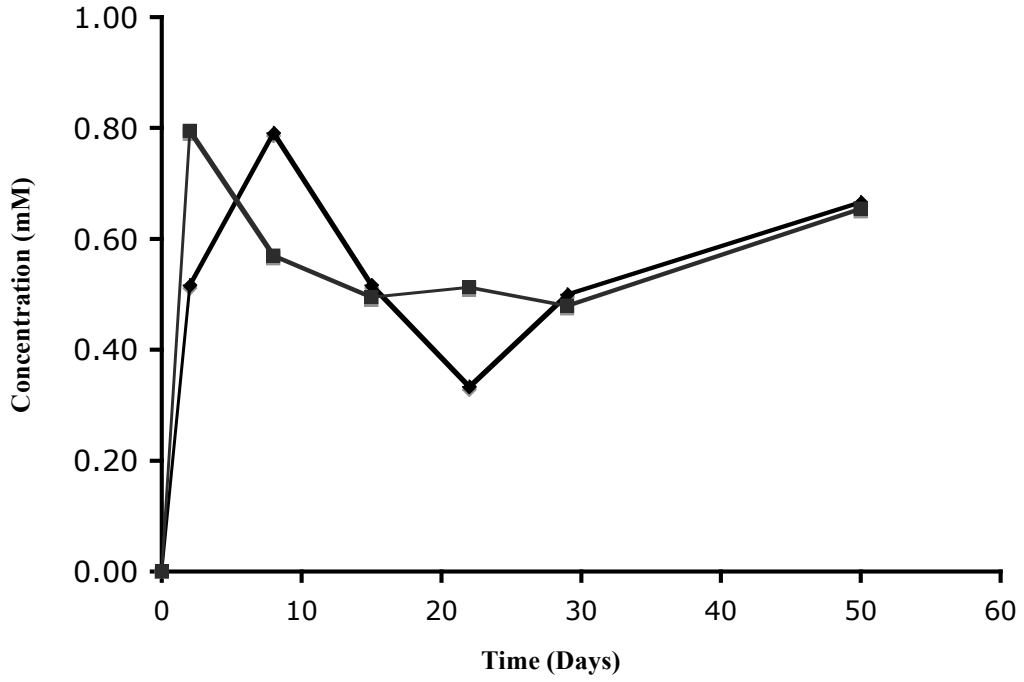


Y: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain M69 in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

Z: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain FX10.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0	0.00	0.00
2	33.07	33.07	0.00	0.80
8	50.67	35.90	23.47	2.42
15	33.07	31.22	1.85	0.00
22	21.33	22.87	0.00	0.00
29	32.00	29.62	2.00	0.97
50	42.67	41.16	1.68	0.46

[1]Values are means of triplicate fermentations

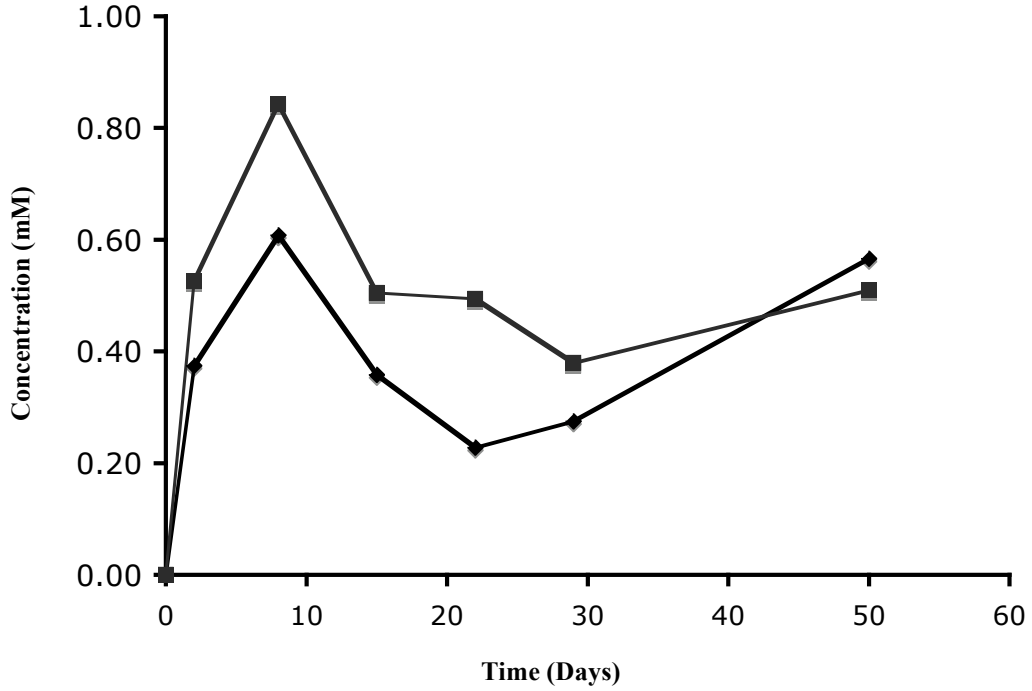


AA: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain FX10 in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

BB: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain S6U.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	24.00	24.52	0.00	0.00
8	38.93	38.64	0.00	0.00
15	22.93	22.88	0.00	0.00
22	14.61	14.71	0.00	0.00
29	17.60	17.93	0.00	0.00
50	36.27	31.84	2.63	1.30

[1]Values are means of triplicate fermentations

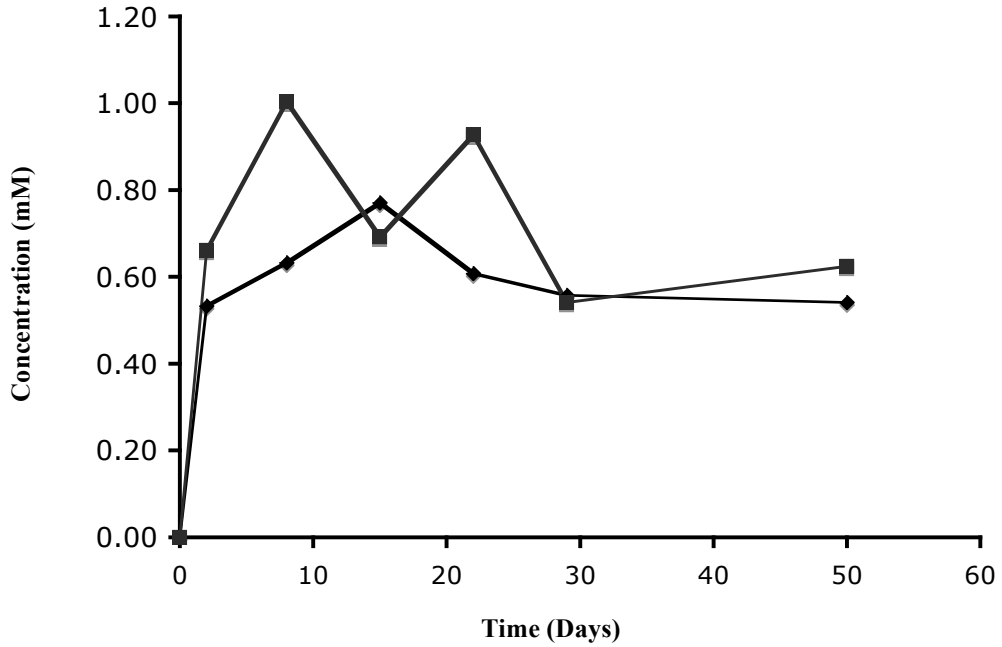


CC: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain S6U in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

DD: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain F15.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	34.13	34.87	0.00	0.00
8	40.53	40.86	0.00	0.00
15	49.39	43.57	6.72	2.51
22	38.93	38.46	0.00	0.00
29	35.73	33.88	2.98	0.05
50	34.67	34.98	0.00	0.00

[1]Values are means of triplicate fermentations

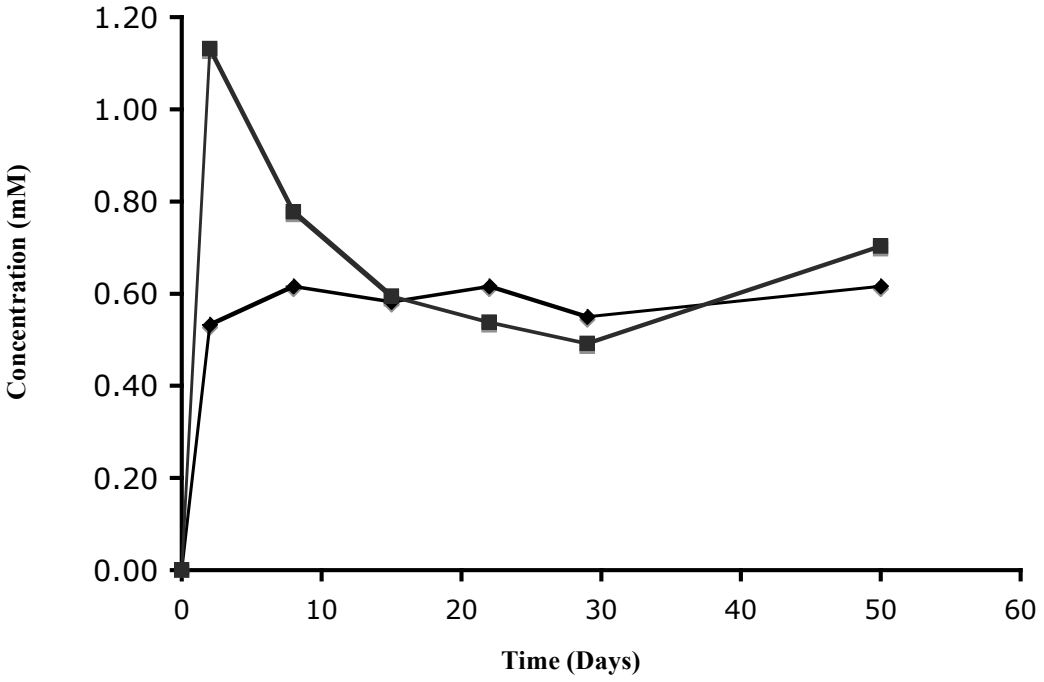


EE: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain S6U in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

FF: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain BM45.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	34.13	36.72	0.00	0.00
8	39.47	39.89	0.00	0.00
15	37.33	37.33	0.00	0.00
22	39.47	34.02	3.51	2.77
29	35.20	30.97	5.37	1.98
50	39.47	39.96	0.00	0.00

[1]Values are means of triplicate fermentations

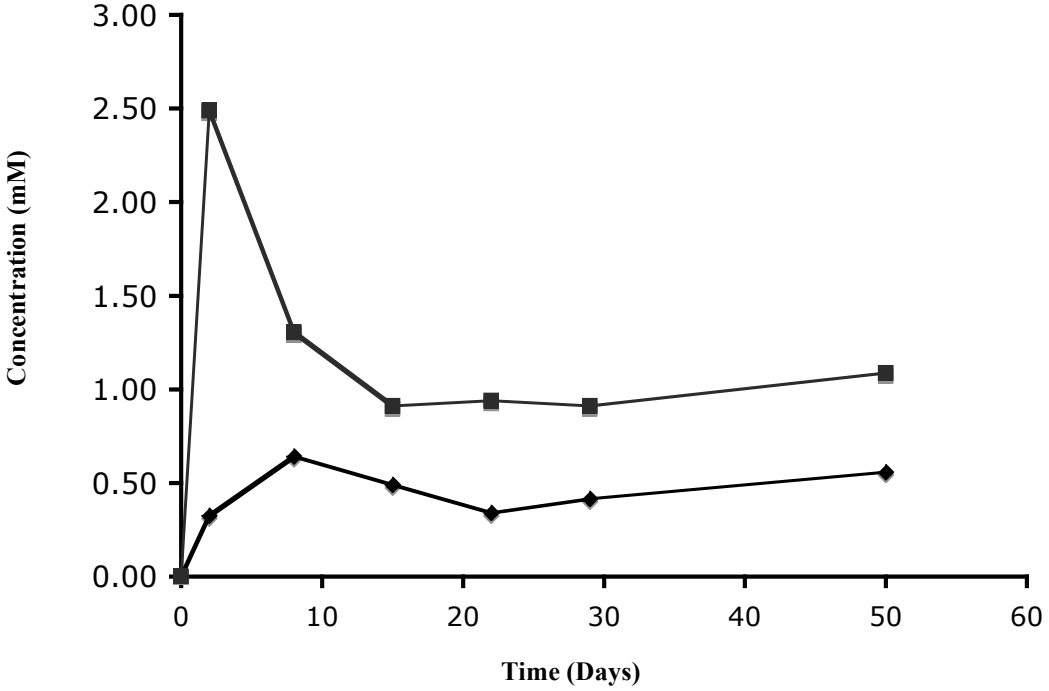


GG: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain BM45 in a synthetic grape juice during the alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

HH: Bound SO_2 and calculated binding compounds (mg/L) in synthetic grape juice during alcoholic fermentation for *S. cerevisiae* strain 43.

Days of Alcoholic Fermentation	Measured Bound SO_2[1] (mg/L)	Calculated Binding Compounds (mg/L)		
		Acetaldehyde	Pyruvic Acid	α-ketoglutarate
0	0.00	0.00	0.00	0.00
2	20.80	20.10	0.00	0.00
8	41.07	40.05	0.00	0.00
15	31.36	31.60	0.00	0.00
22	21.87	21.57	0.00	0.00
29	26.67	26.29	0.00	0.00
50	35.73	35.82	0.00	0.00

[1]Values are means of triplicate fermentations



II: Concentration of bound SO_2 and total acetaldehyde (mM) for *S. cerevisiae* strain 43 in a synthetic grape juice during alcoholic fermentation. Acetaldehyde ■; Bound SO_2 ◆. Values are means of triplicate fermentations.

APPENDIX B

A: Free and bound SO_2 in Hood media (pH 3.5 and 3.7) after addition of SO_2 (mg/L). Results are averages of triplicate samples.

SO_2 added (mg/L)	pH 3.5		pH 3.7	
	Free (mg/L)	**Bound (mg/L)**	**Free (mg/L)**	**Bound (mg/L)**
5	3.2	1.9	3.7	0.0
10	6.4	6.4	6.4	1.6
15	12.8	6.1	12.5	3.1
20	12.7	10.6	18.2	1.4
25	11.2	17.6	18.4	11.0
50	34.2	14.4	45.0	5.0

B: Free and bound SO_2 in Hood media (pH 3.5 and 3.7) after addition of SO_2 bound acetaldehyde (mg/L). Results are averages of triplicate samples.

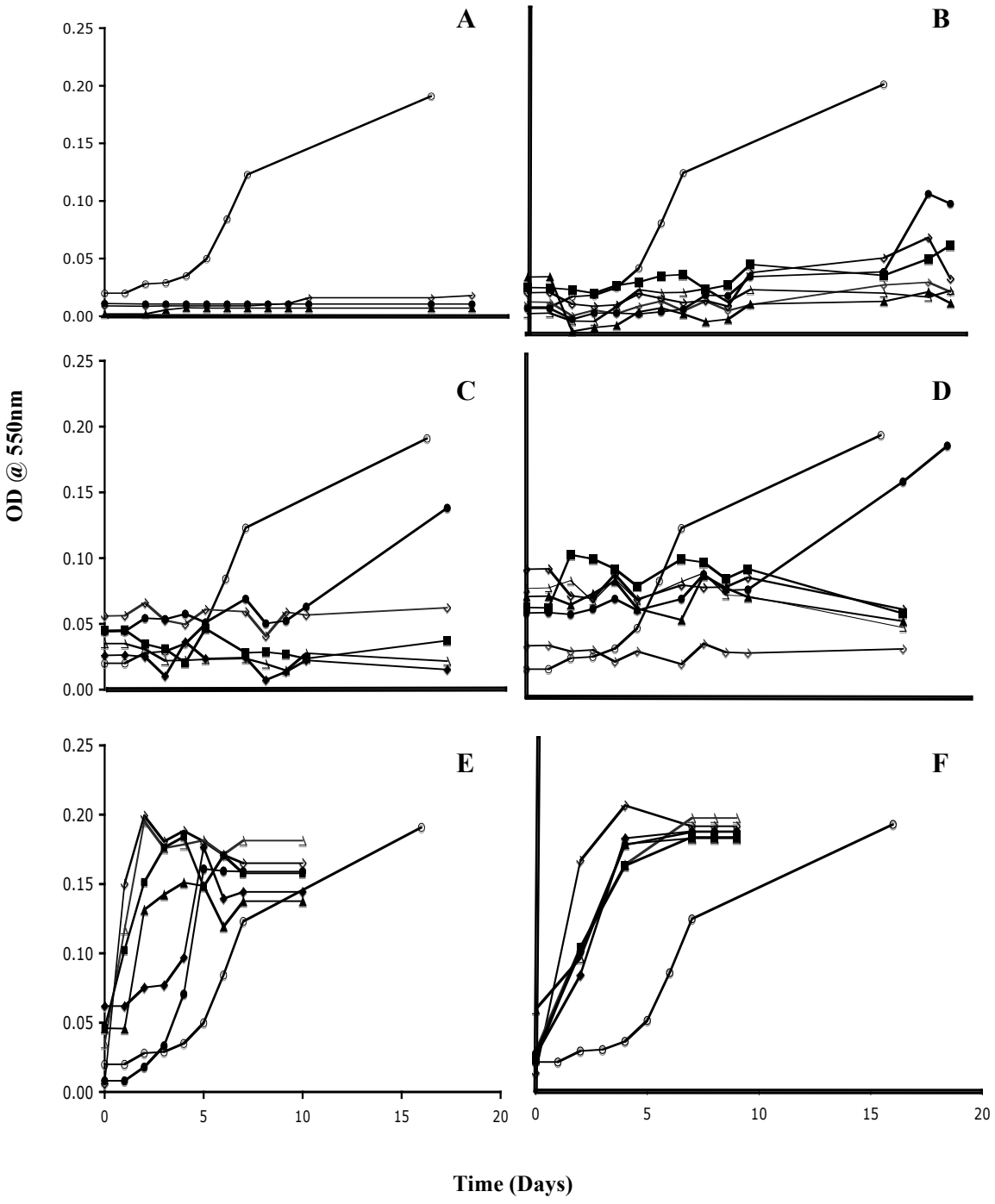
SO_2 added (mg/L)	pH 3.5		pH 3.7	
	Free (mg/L)	**Bound (mg/L)**	**Free (mg/L)**	**Bound (mg/L)**
5	1.6	8.6	0.8	4.3
10	0.0	11.8	0.0	11.8
15	0.0	17.6	0.0	15.0
20	0.0	38.4	0.0	19.3
25	0.0	24.0	0.0	23.9
50	0.0	49.6	1.6	44.8

C: Free and bound SO_2 in Hood media (3.5 and 3.7) after addition of SO_2 bound pyruvic acid (mg/L). Results are averages of triplicate samples.

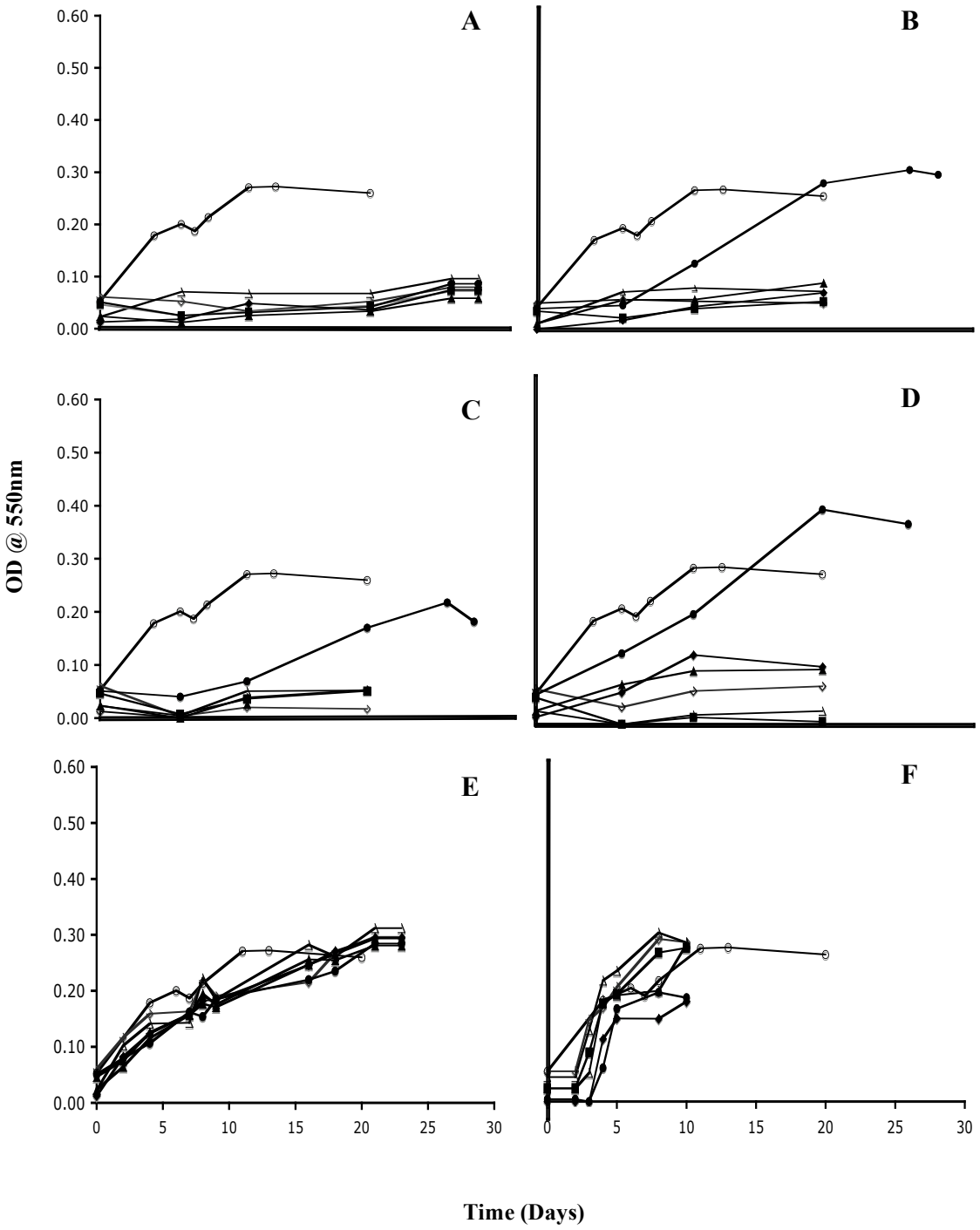
SO_2 added (mg/L)	pH 3.5		pH 3.7	
	Free (mg/L)	**Bound (mg/L)**	**Free (mg/L)**	**Bound (mg/L)**
5	0.8	5.4	0.8	5.4
10	1.7	8.3	8.0	8.3
15	6.4	12.1	6.4	12.1
20	9.6	11.8	9.6	11.8
25	10.2	19.8	10.2	19.8
50	16.0	26.2	16.0	26.1

D: Free and bound SO_2 in Hood media (pH 3.5 and 3.7) after addition of SO_2 bound acetaldehyde and pyruvic acid (mg/L). Results are averages of triplicate samples.

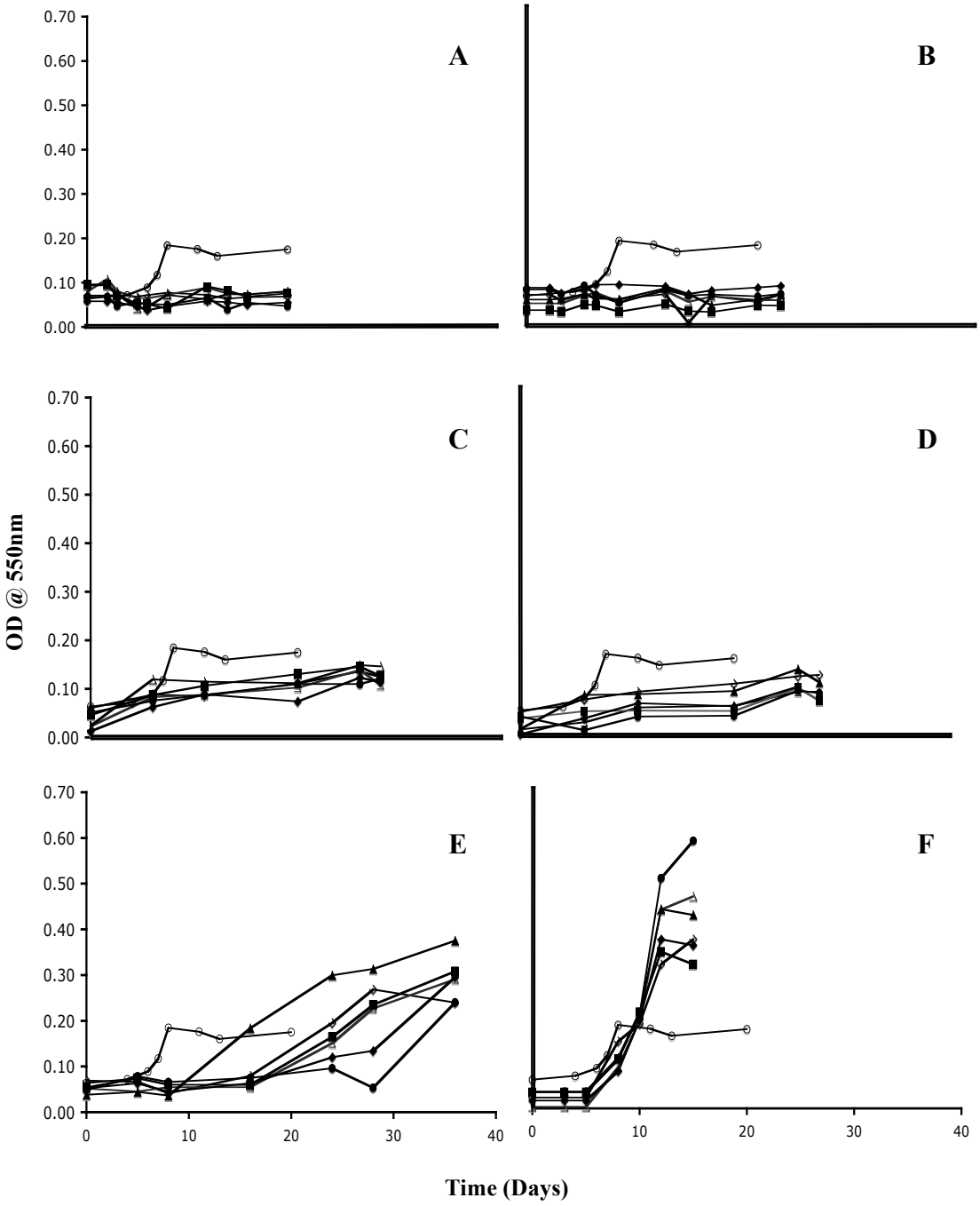
SO_2 added (mg/L)	pH 3.5		pH 3.7	
	Free (mg/L)	**Bound (mg/L)**	**Free (mg/L)**	**Bound (mg/L)**
5	0.0	5.1	0.0	5.1
10	0.0	13.8	0.0	13.8
15	0.2	14.4	0.2	14.4
20	0.2	22.4	0.2	22.4
25	1.6	25.0	1.6	25.0
50	1.6	44.8	1.6	44.8



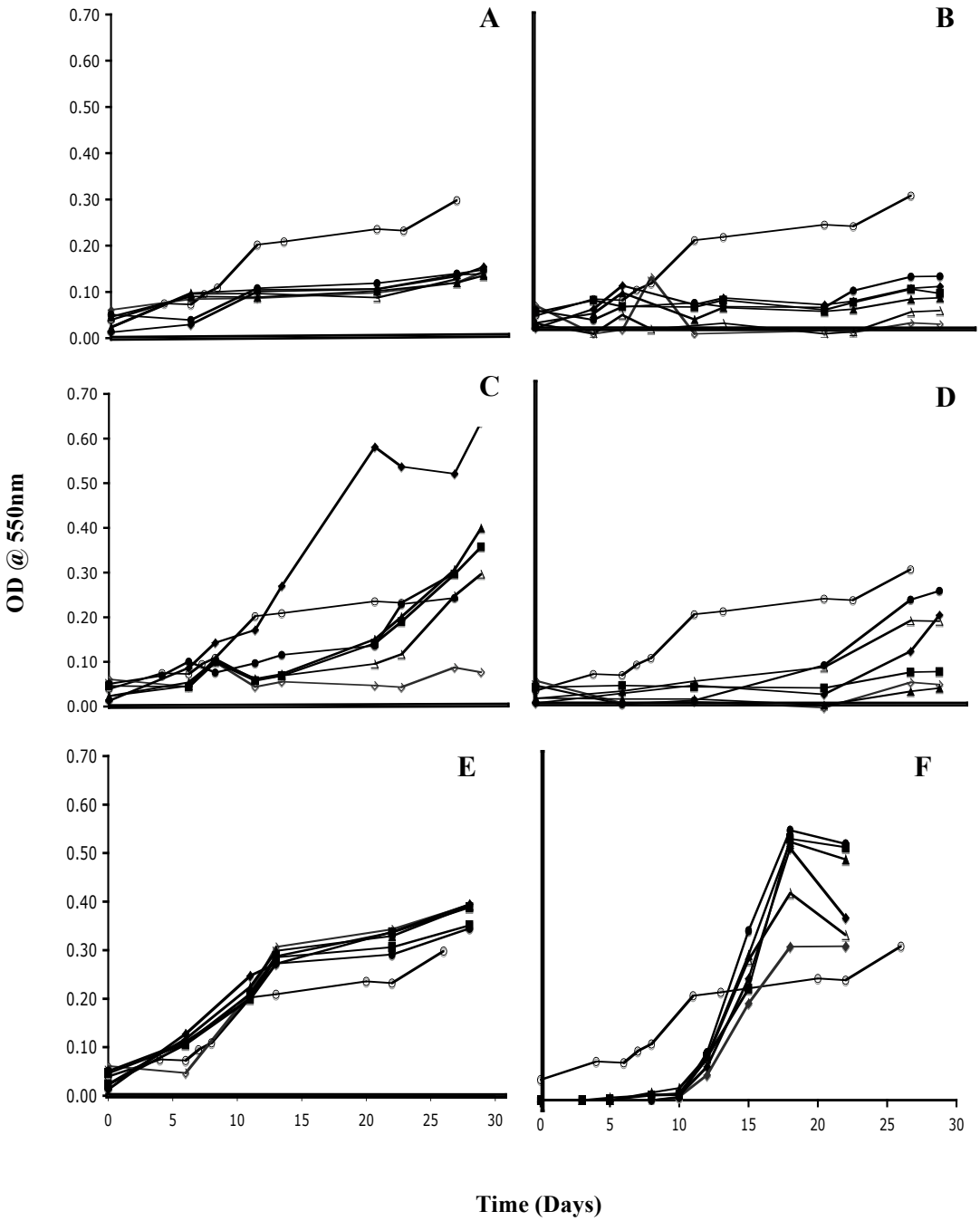
E: Growth of *O. oeni VFO* at 550nm in Hood Media at pH 3.5 containing 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇ of SO_2 (A), SO_2 bound acetaldehyde (B), SO_2 bound pyruvic acid (C), or SO_2 bound acetaldehyde and pyruvic acid (D). Along with growth in media containing either acetaldehyde (E) 0mg/L ○; 8 mg/L ●; 14.5 mg/L ◆; 21 mg/L ▲; 30 mg/L ■; 36 mg/L △; or 72 mg/L ◇ or pyruvic acid (F) at 0mg/L ○; 3.5 mg/L ●; 7 mg/L ◆; 10 mg/L ▲; 15 mg/L ■; 18 mg/L △; or 36 mg/L ◇. Values are means of triplicate samples.



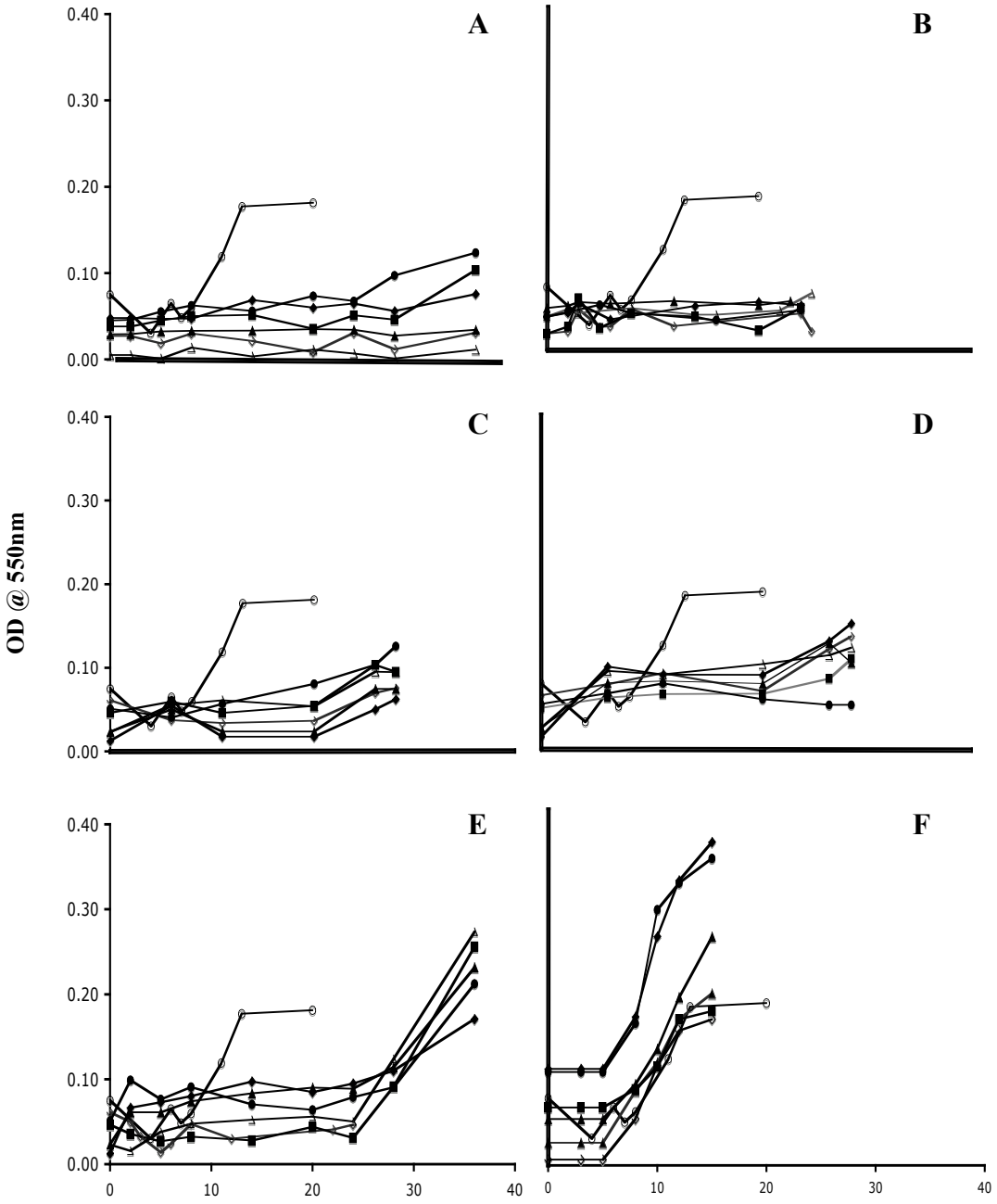
F: Growth of *O. oeni VFO* at 550nm in Hood Media at pH 3.7 containing 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇ of SO_2 (A), SO_2 bound acetaldehyde (B), SO_2 bound pyruvic acid (C), or SO_2 bound acetaldehyde and pyruvic acid (D). Along with growth in media containing either acetaldehyde (E) 0mg/L ○; 8 mg/L ●; 14.5 mg/L ◆; 21 mg/L ▲; 30 mg/L ■; 36 mg/L △; or 72 mg/L ◇ or pyruvic acid (F) at 0mg/L ○; 3.5 mg/L ●; 7 mg/L ◆; 10 mg/L ▲; 15 mg/L ■; 18 mg/L △; or 36 mg/L ◇. Values are means of triplicate samples.



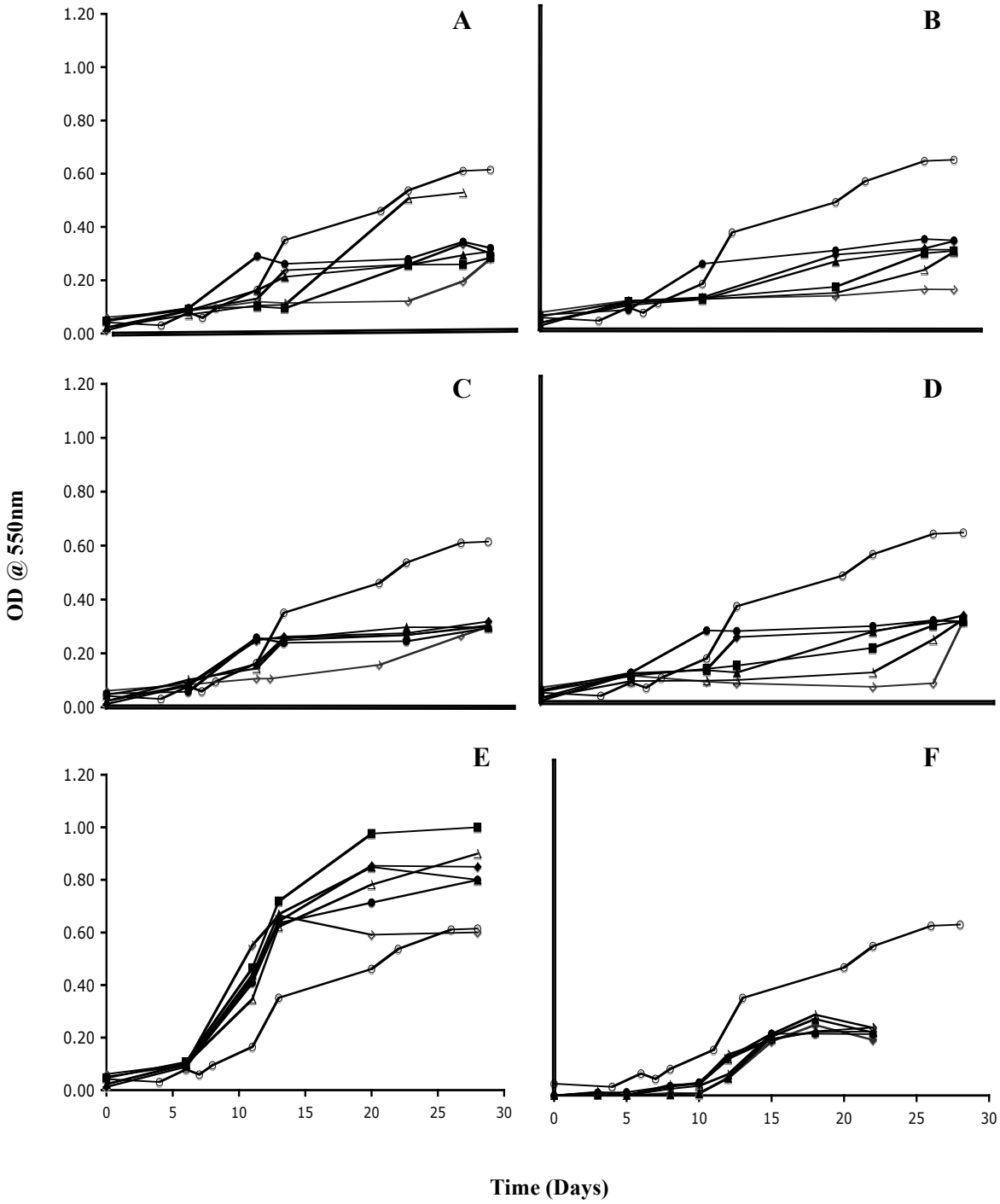
G: Growth of *P. parvulus* at 550nm in Hood Media at pH 3.5 containing 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇ of SO_2 (A), SO_2 bound acetaldehyde (B), SO_2 bound pyruvic acid (C), or SO_2 bound acetaldehyde and pyruvic acid (D). Along with growth in media containing either acetaldehyde (E) 0mg/L ○; 8 mg/L ●; 14.5 mg/L ◆; 21 mg/L ▲; 30 mg/L ■; 36 mg/L △; or 72 mg/L ◇ or pyruvic acid (F) at 0mg/L ○; 3.5 mg/L ●; 7 mg/L ◆; 10 mg/L ▲; 15 mg/L ■; 18 mg/L △; or 36 mg/L ◇. Values are means of triplicate samples.



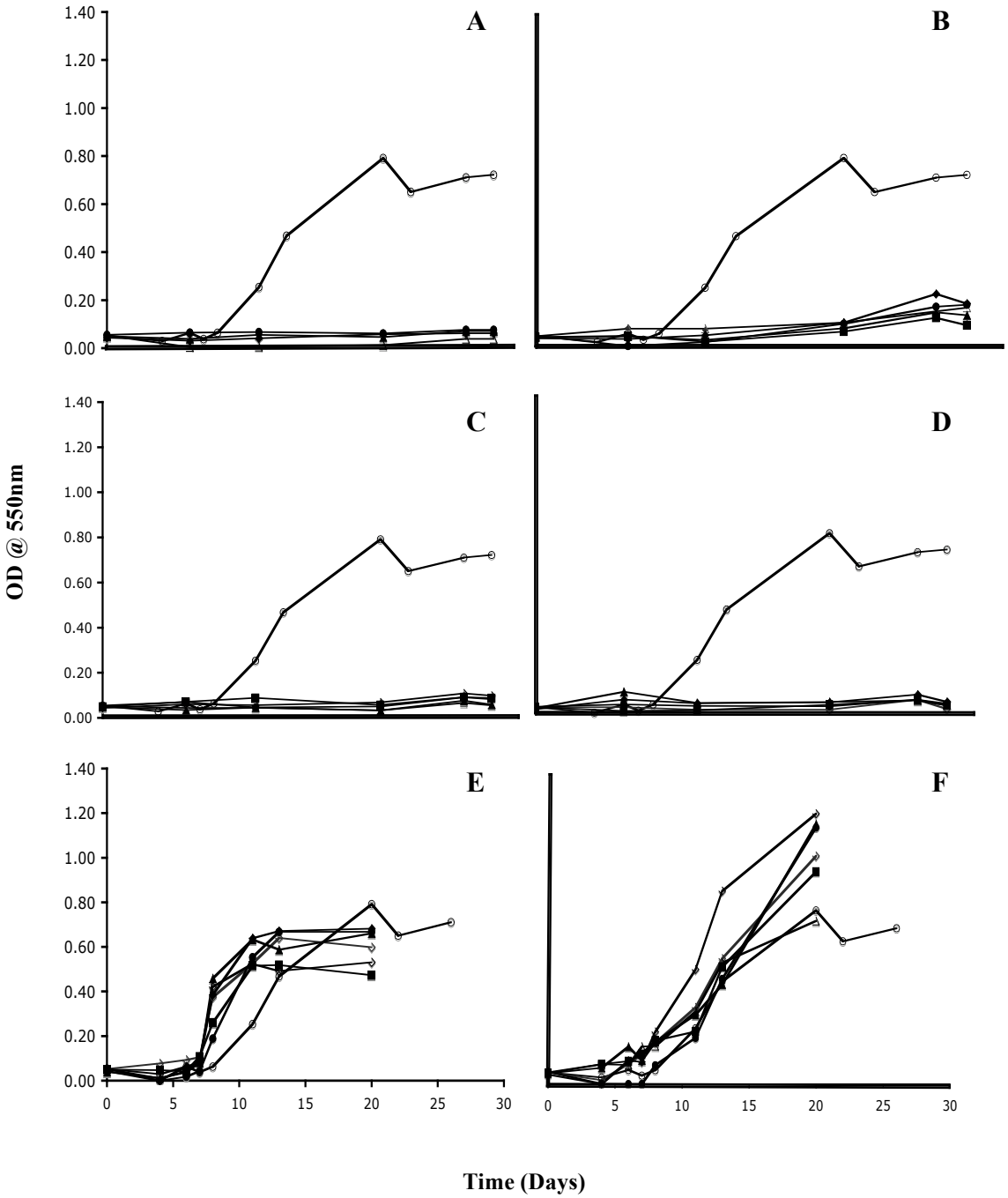
H: Growth of *P. parvulus* at 550nm in Hood Media at pH 3.7 containing 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇ of SO_2 (A), SO_2 bound acetaldehyde (B), SO_2 bound pyruvic acid (C), or SO_2 bound acetaldehyde and pyruvic acid (D). Along with growth in media containing either acetaldehyde (E) 0mg/L ○; 8 mg/L ●; 14.5 mg/L ◆; 21 mg/L ▲; 30 mg/L ■; 36 mg/L △; or 72 mg/L ◇ or pyruvic acid (F) at 0mg/L ○; 3.5 mg/L ●; 7 mg/L ◆; 10 mg/L ▲; 15 mg/L ■; 18 mg/L △; or 36 mg/L ◇. Values are means of triplicate samples.



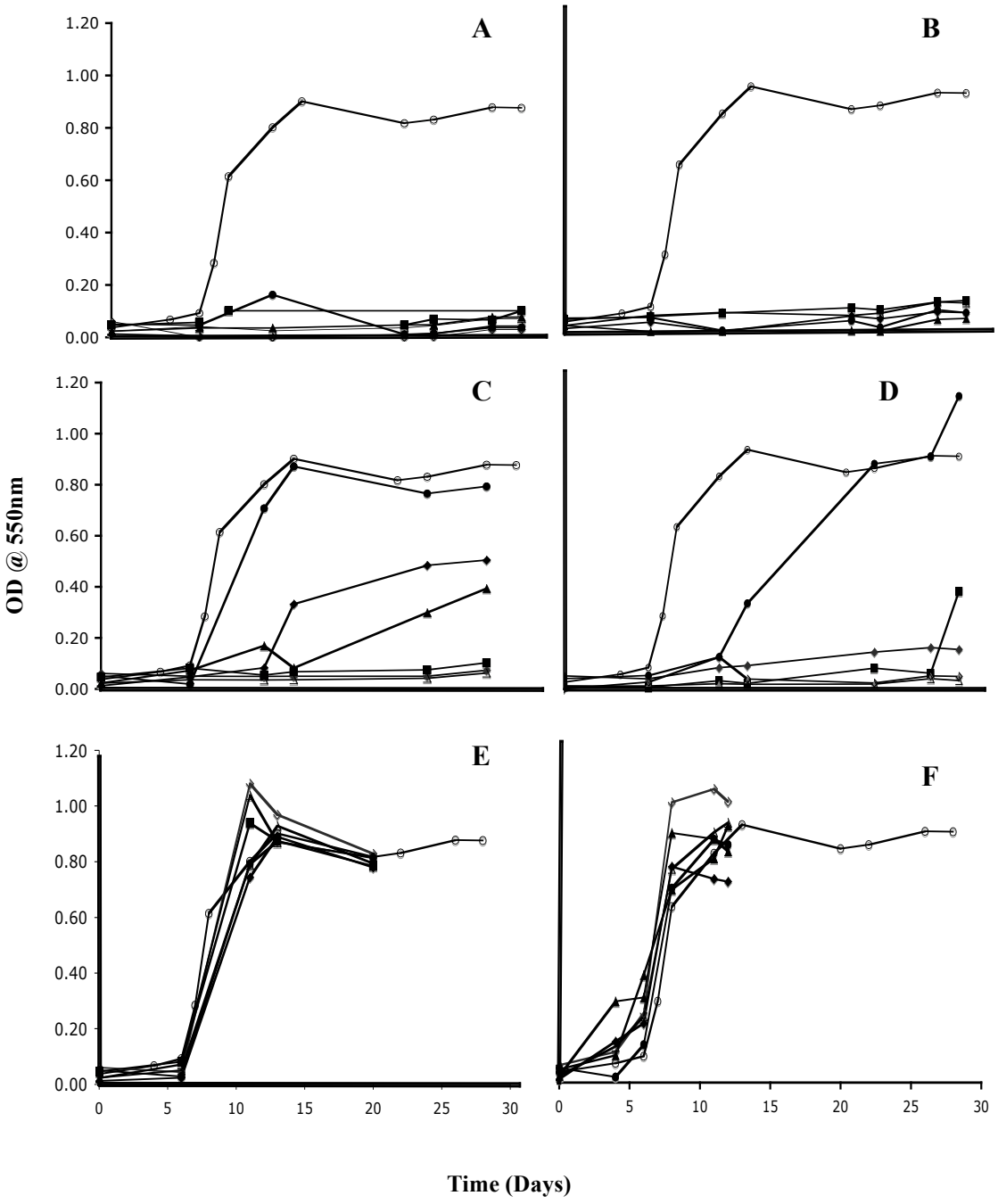
I: Growth of *P. damnosus* at 550nm in Hood Media at pH 3.5 containing 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇ of SO_2 (A), SO_2 bound acetaldehyde (B), SO_2 bound pyruvic acid (C), or SO_2 bound acetaldehyde and pyruvic acid (D). Along with growth in media containing either acetaldehyde (E) 0mg/L ○; 8 mg/L ●; 14.5 mg/L ◆; 21 mg/L ▲; 30 mg/L ■; 36 mg/L △; or 72 mg/L ◇ or pyruvic acid (F) at 0mg/L ○; 3.5 mg/L ●; 7 mg/L ◆; 10 mg/L ▲; 15 mg/L ■; 18 mg/L △; or 36 mg/L ◇. Values are means of triplicate samples.



J: Growth of *P. damnosus* at 550nm in Hood Media at pH 3.7 containing 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇ of SO_2 (A), SO_2 bound acetaldehyde (B), SO_2 bound pyruvic acid (C), or SO_2 bound acetaldehyde and pyruvic acid (D). Along with growth in media containing either acetaldehyde (E) 0mg/L ○; 8 mg/L ●; 14.5 mg/L ◆; 21 mg/L ▲; 30 mg/L ■; 36 mg/L △; or 72 mg/L ◇ or pyruvic acid (F) at 0mg/L ○; 3.5 mg/L ●; 7 mg/L ◆; 10 mg/L ▲; 15 mg/L ■; 18 mg/L △; or 36 mg/L ◇. Values are means of triplicate samples.



K: Growth of *Lb. hilgardii* at 550nm in Hood Media at pH 3.5 containing 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇ of SO_2 (A), SO_2 bound acetaldehyde (B), SO_2 bound pyruvic acid (C), or SO_2 bound acetaldehyde and pyruvic acid (D). Along with growth in media containing either acetaldehyde (E) 0mg/L ○; 8 mg/L ●; 14.5 mg/L ◆; 21 mg/L ▲; 30 mg/L ■; 36 mg/L △; or 72 mg/L ◇ or pyruvic acid (F) at 0mg/L ○; 3.5 mg/L ●; 7 mg/L ◆; 10 mg/L ▲; 15 mg/L ■; 18 mg/L △; or 36 mg/L ◇. Values are means of triplicate samples.



L: Growth of *Lb. hilgardii* at 550nm in Hood Media at pH 3.7 containing 0mg/L ○; 5mg/L ●; 10mg/L ◆; 15mg/L ▲; 20mg/L ■; 25mg/L △; or 50mg/L ◇ of SO_2 (A), SO_2 bound acetaldehyde (B), SO_2 bound pyruvic acid (C), or SO_2 bound acetaldehyde and pyruvic acid (D). Along with growth in media containing either acetaldehyde (E) 0mg/L ○; 8 mg/L ●; 14.5 mg/L ◆; 21 mg/L ▲; 30 mg/L ■; 36 mg/L △; or 72 mg/L ◇ or pyruvic acid (F) at 0mg/L ○; 3.5 mg/L ●; 7 mg/L ◆; 10 mg/L ▲; 15 mg/L ■; 18 mg/L △; or 36 mg/L ◇. Values are means of triplicate samples.

M: Concentrations of acetaldehyde 0, 7, and 14 days after inoculation with *O. oeni* VFO into Hood media containing different initial acetaldehyde (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**
Acetaldehyde (mg/L)	70.35	50.35	0.6528
	34.09	25.92	0.7616
	35.25	20.30	0.9792
	18.92	15.43	1.088
	15.14	10.23	0.8704
	7.92	5.57	0.7616
B			
Acetaldehyde (mg/L)	70.35	0.87	0.00
	34.09	1.20	0.00
	35.25	1.41	1.6
	18.92	0.98	1.09
	15.14	0.98	2.40
	7.92	2.28	1.74

N: Concentrations of pyruvic acid 0 and 7 days after inoculation with *O. oeni* VFO into Hood media containing different initial pyruvic acid (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**
Pyruvic acid (mg/L)	35.94	0.00
	20.04	0.00
	18.76	0.00
	11.59	0.00
	5.02	0.00
	4.56	0.00
B		
Pyruvic acid (mg/L)	35.94	0.00
	20.04	0.00
	18.76	0.00
	11.59	0.00
	5.02	0.00
	3.08	0.00

O: Concentrations of acetaldehyde 0, 7, 14, 21, and 28 days after inoculation with *O. oeni* VFO into Hood media containing different initial SO_2 bound acetaldehyde (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	72.71	41.07	48.73	38.78	39.46
	36.35	19.11	21.4	20.47	20.00
	29.08	13.27	14.22	13.45	12.93
	21.08	12.51	11.14	10.25	10.31
	14.54	6.56	3.26	1.93	1.23
	7.27	1.20	0.21	0.03	0.01
B					
Acetaldehyde (mg/L)	72.32	38.19	24.59	29.81	24.37
	36.45	9.90	13.06	17.63	6.75
	30.02	12.40	10.01	9.25	10.23
	21.38	13.27	6.42	5.33	0.11
	14.50	8.27	2.18	2.94	1.85
	4.39	2.89	1.12	0.00	0.00

P: Concentrations of pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *O. oeni* VFO into Hood media containing different initial SO_2 bound pyruvic acid (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Pyruvic acid (mg/L)	36.12	43.10	47.59	35.53	38.52
	25.15	26.69	25.00	31.79	25.47
	23.23	23.23	21.62	23.75	25.47
	19.38	19.77	19.45	20.99	22.43
	9.69	12.21	11.39	18.46	19.15
	7.86	9.15	10.03	8.80	10.01
B					
Pyruvic acid (mg/L)	45.93	17.01	18.92	17.21	11.05
	27.31	3.49	2.077	4.38	3.36
	25.93	0.79	1.821	2.97	2.33
	22.31	0.00	0.00	2.21	1.56
	20.93	0.00	0.00	0.54	0.00
	18.00	0.00	0.00	0.00	0.00

Q: Concentrations of acetaldehyde and pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *O. oeni* VFO into Hood media containing different initial SO_2 bound acetaldehyde (A) and pyruvic acid (B) (mg/L) concentrations at pH 3.5. Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	35.47	33.91	29.48	27.56	20.27
	14.04	17.63	21.83	16.10	15.99
	14.80	15.7	10.77	12.08	6.13
	10.99	10.37	11.39	2.14	6.20
	7.72	6.93	4.61	10.95	4.06
	4.68	4.10	2.90	0.40	1.56
B					
Pyruvic acid (mg/L)	37.44	39.08	38.00	39.95	44.38
	24.18	21.60	23.00	27.94	28.46
	23.16	21.26	20.00	24.49	26.45
	18.16	13.57	13.57	23.06	24.95
	16.83	13.98	12.00	20.41	20.70
	12.55	12.17	7.31	6.28	8.92

R: Concentrations of acetaldehyde and pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *O. oeni* VFO into Hood media containing different initial SO_2 bound acetaldehyde (A) and pyruvic acid (B) (mg/L) concentrations at pH 3.7. Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	34.82	23.83	24.50	25.50	20.45
	25.35	4.90	10.01	12.95	9.03
	14.14	11.32	7.07	7.51	5.55
	11.75	5.66	8.16	0.11	2.94
	10.00	3.16	3.92	0.98	1.52
	4.13	1.31	1.31	1.41	1.41
B					
Pyruvic acid (mg/L)	44.90	19.92	20.21	19.90	17.85
	29.72	3.23	3.56	4.90	5.79
	26.10	5.67	7.82	6.59	5.41
	25.24	0.00	0.00	0.00	0.00
	22.31	0.00	0.00	0.00	0.00
	19.03	0.00	0.00	0.00	0.00

S: Concentrations of acetaldehyde 0, 7, 14, 21, 28, and 36 days after inoculation with *P. parvulus* into Hood media containing different initial acetaldehyde (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**	**36**
Acetaldehyde (mg/L)	70.35	22.52	18.93	16.25	13.38	9.14
	34.09	9.9	7.14	6.46	5.80	2.61
	35.25	7.22	4.06	3.34	2.54	1.34
	18.92	7.36	2.94	3.45	5.22	0.00
	15.14	4.42	1.96	2.65	2.32	0.00
	7.92	3.16	1.52	2.83	3.12	0.00
B						
Acetaldehyde (mg/L)	70.35	2.07	1.20	2.18	1.85	0.00
	34.09	2.39	1.31	2.50	2.07	0.00
	35.25	3.16	1.96	0.11	1.85	0.00
	18.92	5.33	2.50	3.16	1.63	0.00
	15.14	0.44	1.31	3.37	3.37	0.00
	7.92	0.65	1.08	3.48	3.05	0.00

T: Concentrations of pyruvic acid 0 and 7 days after inoculation with *P. parvulus* into Hood media containing different initial pyruvic acid (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**
Pyruvic acid (mg/L)	35.94	14.26
	20.04	3.87
	18.76	2.46
	11.59	1.56
	5.02	0.00
	4.56	0.00
B		
Pyruvic acid (mg/L)	35.94	7.72
	20.04	0.00
	18.76	0.00
	11.59	0.00
	5.02	0.00
	4.56	0.00

U: Concentrations of acetaldehyde 0, 7, 14, 21, and 28 days after inoculation with *P. parvulus* into Hood media containing different initial SO_2 bound acetaldehyde (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	76.16	50.75	55.42	46.68	56.14
	35.1	28.72	18.71	16.32	16.68
	26.22	15.7	15.09	14.11	10.01
	20.02	11.53	14.58	15.34	15.99
	13.9	8.27	4.79	5.95	6.02
	8	4.93	2.18	3.41	3.92
B					
Acetaldehyde (mg/L)	72.32	22.52	13.93	12.73	8.38
	36.45	11.21	5.22	4.68	2.28
	30.02	7.62	3.70	3.59	2.28
	21.38	3.92	3.48	1.41	2.94
	14.5	4.24	2.61	1.85	2.18
	8.98	2.28	1.31	2.39	2.83

V: Concentrations of pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *P. parvulus* into Hood media containing different initial SO_2 bound pyruvic acid (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Pyruvic acid (mg/L)	45.93	14.13	10.67	15.54	10.32
	27.31	2.85	3.23	6.44	3.02
	25.93	2.21	3.87	5.79	1.20
	22.31	1.05	2.46	1.69	0.01
	20.93	0.00	0.00	0.00	0.00
	18.00	0.03	0.00	0.00	0.00
B					
Pyruvic acid (mg/L)	45.93	9.64	7.59	12.08	17.33
	27.31	2.72	1.95	3.36	0.00
	25.93	1.69	0.28	16.69	0.00
	22.31	0.15	0.00	1.31	0.00
	20.93	0.00	0.00	0.00	0.00
	18.00	0.00	0.00	0.00	0.00

W: Concentrations of acetaldehyde and pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *P. parvulus* into Hood media containing different initial SO_2 bound acetaldehyde (A) and pyruvic acid (B) (mg/L) concentrations at pH 3.5. Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	34.82	30.03	28.94	23.28	0.00
	25.35	16.65	8.05	10.12	0.44
	14.14	13.49	14.04	10.34	2.28
	11.75	7.83	4.13	7.29	0.00
	10.00	6.31	4.46	4.46	3.48
	4.13	2.61	0.87	2.39	0.87
B					
Pyruvic acid (mg/L)	44.90	12.59	10.92	11.56	9.22
	29.72	8.74	5.67	7.85	4.02
	26.10	0.00	0.00	4.00	1.20
	25.24	0.00	1.44	0.00	0.01
	22.31	0.00	0.00	0.00	0.00
	19.03	0.00	0.00	0.00	0.00

X: Concentrations of acetaldehyde and pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *P. parvulus* into Hood media containing different initial SO_2 bound acetaldehyde (A) and pyruvic acid (B) (mg/L) concentrations at pH 3.7. Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	34.82	18.50	12.30	9.79	7.51
	25.35	10.44	4.46	2.39	1.96
	14.14	6.53	2.94	2.50	0.00
	11.75	0.00	2.82	2.83	1.20
	10.00	0.11	0.97	1.31	1.52
	4.13	1.85	1.31	1.52	0.98
B					
Pyruvic acid (mg/L)	44.90	22.64	12.72	13.62	22.72
	29.72	4.26	7.08	5.28	6.18
	26.10	1.56	4.00	2.85	5.03
	25.24	2.21	2.46	1.69	3.36
	22.31	0.00	0.00	0.00	0.00
	19.03	15.00	12.72	0.00	0.00
	44.90	22.64	12.72	13.62	22.72

Y: Concentrations of acetaldehyde 0, 7,14, 21, 28, and 36 days after inoculation with *P. damnosus* into Hood media containing different initial acetaldehyde (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**	**36**
Acetaldehyde (mg/L)	70.35	20.06	12.91	11.79	10.99	7.62
	34.09	13.85	8.31	9.97	4.61	1.81
	35.25	11.35	6.67	8.01	7.51	0.76
	18.92	7.72	4.90	5.40	2.50	3.05
	15.14	5.73	4.17	4.24	3.92	0.36
	7.92	4.32	3.16	3.37	1.96	0.94
B						
Acetaldehyde (mg/L)	70.35	2.07	2.39	0.00	0.00	0.00
	34.09	2.39	1.41	0.00	0.00	0.00
	35.25	3.16	1.63	0.00	0.00	0.00
	18.92	5.33	2.39	1.22	0.00	0.00
	15.14	0.44	0.00	0.00	0.00	0.00
	7.92	0.65	1.09	0.00	0.00	0.00

Z: Concentrations of acetaldehyde 0, 7, and 14 days after inoculation with *P. damnosus* into Hood media containing different initial pyruvic acid (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**
Pyruvic acid (mg/L)	35.94	16.05	0.00
	20.04	2.21	0.00
	18.76	5.41	0.00
	11.59	0.00	0.00
	5.02	0.00	0.00
	4.56	0.00	0.00
B			
Pyruvic acid (mg/L)	35.94	11.95	0.00
	20.04	5.15	0.00
	18.76	3.62	0.00
	11.59	0.00	0.00
	5.02	0.00	0.00
	4.56	0.00	0.00

AA: Concentrations of acetaldehyde 0, 7, 14, 21, 28, and 36 days after inoculation with *P. damnosus* into Hood media containing different initial SO_2 bound acetaldehyde (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

	0	**7**	**14**	**21**	**28**	**36**
Acetaldehyde (mg/L)	76.16	48.3	45.73	45.73	42.58	38.15
	35.10	24.12	23.97	21.83	24.88	16.61
	26.22	17.55	14.40	9.10	10.44	8.78
	20.02	14.36	11.68	9.68	10.81	7.25
	13.90	9.60	8.60	8.20	8.23	4.28
	8.00	4.24	3.05	3.59	3.52	1.23
B						
Acetaldehyde (mg/L)	72.32	25.35	11.75	11.97	12.40	0.00
	36.45	8.92	5.77	3.48	2.61	0.00
	30.02	10.99	2.94	2.83	1.63	0.00
	21.38	5.44	3.48	1.20	2.83	0.00
	14.50	3.37	1.74	0.76	2.28	0.00
	8.98	2.07	1.63	0.76	3.48	0.00

BB: Concentrations of pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *P. damnosus* into Hood media containing different initial SO_2 bound pyruvic acid (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Pyruvic acid (mg/L)	45.93	17.21	15.92	9.51	2.09
	27.31	4.13	5.03	4.77	1.67
	25.93	1.82	3.36	7.21	3.83
	22.31	0.00	2.85	2.59	1.23
	20.93	0.00	0.00	0.00	0.00
	18.00	0.00	0.00	0.00	0.00
B					
Pyruvic acid (mg/L)	45.93	13.10	14.26	0.00	0.00
	27.31	0.67	0.00	0.00	0.00
	25.93	0.00	0.00	0.00	0.00
	22.31	0.00	0.00	0.00	0.00
	20.93	0.00	0.00	0.00	0.00
	18.00	0.00	0.00	0.00	0.00

CC: Concentrations of acetaldehyde and pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *P. damnosus* into Hood media containing different initial SO_2 bound acetaldehyde (A) and pyruvic acid (B) (mg/L) concentrations at pH 3.5. Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	72.32	30.14	24.26	31.23	19.04
	36.45	12.95	8.27	13.49	0.00
	30.02	11.53	10.55	8.49	5.33
	21.38	7.72	9.14	4.90	6.53
	14.50	4.35	4.03	7.29	1.74
	8.98	2.18	1.52	2.61	0.22
B					
Pyruvic acid (mg/L)	44.90	15.92	14.26	11.95	8.43
	29.72	5.41	5.41	5.54	2.59
	26.10	2.08	0.00	2.33	0.53
	25.24	0.67	0.00	0.00	0.00
	22.31	0.00	0.00	0.00	0.00
	19.03	0.00	14.26	0.00	0.00

DD: Concentrations of acetaldehyde and pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *P. damnosus* into Hood media containing different initial SO_2 bound acetaldehyde (A) and pyruvic acid (B) (mg/L) concentrations at pH 3.7. Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	34.82	21.54	15.88	11.42	8.38
	25.25	8.70	5.88	3.92	1.09
	14.14	7.18	2.94	2.61	1.85
	11.75	5.55	3.37	3.16	1.41
	10.12	0.00	0.65	1.41	1.31
	4.13	1.52	1.63	1.63	1.52
B					
Pyruvic acid (mg/L)	44.90	34.92	20.98	16.31	13.74
	29.72	4.90	6.82	0.00	0.00
	26.10	2.85	4.13	0.00	0.00
	25.24	0.15	0.41	0.00	0.00
	22.31	0.00	0.00	0.00	0.00
	19.03	0.00	0.00	0.00	0.00

EE: Concentrations of acetaldehyde 0, 7, and 14 days after inoculation with *Lb. hilgardii* into Hood media containing different initial acetaldehyde (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**
Acetaldehyde (mg/L)	70.35	1.41	0.00
	34.09	1.96	0.44
	35.25	1.20	1.52
	18.92	1.74	1.09
	15.14	2.18	0.65
	7.92	0.00	0.98
B			
Acetaldehyde (mg/L)	70.35	2.94	2.06
	34.09	0.00	1.52
	35.25	1.10	2.61
	18.92	0.00	2.06
	15.14	2.28	0.33
	7.92	0.97	2.61

FF: Concentrations of pyruvic acid 0 and 7 days after inoculation with *Lb. hilgardii* into Hood media containing different initial pyruvic acid (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**
Pyruvic acid (mg/L)	35.94	18.36
	20.04	0.00
	18.76	1.05
	11.59	0.00
	5.02	0.00
	4.56	0.00
B		
Pyruvic acid (mg/L)	35.94	7.03
	20.04	4.85
	18.76	7.79
	11.59	7.92
	5.02	6.26
	4.56	6.77

GG: Concentrations of acetaldehyde 0, 7, 14, 21, and 28 days after inoculation with *Lb. hilgardii* into Hood media containing different initial SO_2 bound acetaldehyde (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	72.32	48.31	36.12	33.73	36.99
	36.45	18.50	13.06	14.58	8.81
	30.02	12.73	9.79	1.52	4.57
	21.38	11.21	10.88	6.75	5.55
	14.5	13.16	5.11	10.88	1.52
	8.98	5.44	1.63	6.75	4.57
B					
Acetaldehyde (mg/L)	45.93	13.10	14.26	0.00	0.00
	27.31	0.67	0.00	0.00	0.00
	25.93	0.00	0.00	0.00	0.00
	22.31	0.00	0.00	0.00	0.00
	20.93	0.00	0.00	0.00	0.00
	18.00	0.00	0.00	0.00	0.00

HH: Concentrations of pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *Lb. hilgardii* into Hood media containing different initial SO_2 bound pyruvic acid (mg/L) concentrations at pH 3.5 (A) and 3.7 (B). Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Pyruvic acid (mg/L)	45.93	14.38	11.56	19.26	19.38
	27.31	1.69	1.82	5.03	4.13
	25.93	1.18	15.15	2.72	2.21
	22.31	0.00	0.00	1.44	0.54
	20.93	0.00	0.00	0.00	0.00
	18.00	0.00	0.00	0.00	0.00
B					
Pyruvic acid (mg/L)	45.93	7.21	8.23	15.67	17.59
	27.31	1.56	1.69	5.15	7.97
	25.93	0.00	0.00	6.44	1.95
	22.31	0.00	0.00	1.82	0.00
	20.93	0.00	0.00	0.00	0.00
	18.00	0.00	0.00	0.00	0.00

II: Concentrations of acetaldehyde and pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *Lb. hilgardii* into Hood media containing different initial SO_2 bound acetaldehyde (A) and pyruvic acid (B) (mg/L) concentrations at pH 3.5. Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	34.82	28.32	25.024	28.29	0.00
	25.25	14.57	10.662	9.25	0.00
	14.14	11.96	9.248	4.57	0.00
	11.75	9.17	5.331	6.53	3.26
	10.12	9.16	8.109	6.20	0.00
	4.13	2.83	2.11	2.94	3.26
B					
Pyruvic acid (mg/L)	44.90	11.44	15.15	21.18	12.33
	29.72	2.72	2.85	7.21	5.67
	26.10	0.00	4.77	3.23	1.95
	25.24	0.00	1.18	0.79	0.15
	22.31	0.00	0.54	0.00	0.00
	19.03	0.00	0.00	0.00	0.00

JJ: Concentrations of acetaldehyde and pyruvic acid 0, 7, 14, 21, and 28 days after inoculation with *Lb. hilgardii* into Hood media containing different initial SO_2 bound acetaldehyde (A) and pyruvic acid (B) (mg/L) concentrations at pH 3.7. Values are means of triplicate samples.

A	**0**	**7**	**14**	**21**	**28**
Acetaldehyde (mg/L)	34.82	24.59	20.70	4.13	23.28
	25.25	12.08	6.09	15.12	13.82
	14.14	8.81	6.31	4.78	3.59
	11.75	9.14	5.11	8.92	5.11
	10.12	6.20	2.83	2.61	2.72
	4.13	4.24	1.96	1.74	1.52
B					
Pyruvic acid (mg/L)	44.90	16.56	22.08	15.67	19.51
	29.72	2.33	5.92	7.85	6.95
	26.10	0.03	6.18	5.54	4.26
	25.24	0.00	0.41	5.67	0.54
	22.31	0.00	0.00	0.00	0.00
	19.03	0.00	0.00	0.00	0.00