

## SPOILAGE MICROBES AND THEIR DETECTION: WHAT IS NEW AND WHAT HAS CHANGED?

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### Introduction

Some believe that the incidence of spoilage microorganisms in wines has increased in recent years. Though not all winemakers agree with this speculation, two issues of potential importance are increases in must pH and a wider application of improved detection methods.

Winemakers have experimented with delaying fruit harvest as a means to favorably alter flavor and tannin profiles. In many cases, wine quality has improved. However, one consequence of this practice is that grapes frequently suffer from higher pH, a condition favorable to the growth of various microorganisms (Davis *et al.*, 1986a). The pH of musts also influences the effectiveness of SO<sub>2</sub> with less amounts of the antimicrobial portion (molecular) present at higher pH. As such, some have suggested that the increased "hang-time" prior to harvest can also adversely impact wine quality by encouraging the potential for microbial spoilage.

Another issue has been application of improved methods to detect microorganisms in musts and wines. Perhaps the most important are the so-called "real-time" molecular techniques. Based upon determination of similarities at the gene level, these methods are being used to identify and, in some cases, quantify microbiological populations. While these methods offer tremendous opportunities to improve microbiological control during vinification, there are potential limitations to routinely using these methods in wine analysis.

This paper summarizes the impact of selected spoilage microorganisms, namely *Brettanomyces*, *Pediococcus*, and *Lactobacillus*, on wine quality as well as some methods for detection.

### *Brettanomyces*

The yeasts *Brettanomyces/Dekkera* are well-known wine spoilage microorganisms whose growth can result in haziness or production of off-odors sometimes described as 'medicinal,' 'mousiness,' 'Band-aid®,' 'barnyard,' or others (Gilliland, 1961; Heresztyn, 1986; Fugelsang *et al.*, 1993; Sponholz, 1993). Previously described species of *Brettanomyces* isolated from wines have been reclassified several times, with *D. bruxellensis* and *D. anomala* now believed to be the microorganisms associated with wine spoilage (Grbin and Henschke, 2000).

While many wine microorganisms including *Acetobacter*, *O. oeni*, *L. hilgardii*, *L. plantarum*, *L. brevis*, *P. pentosaceus*, *P. damnosus*, and *Saccharomyces* can synthesize 4-vinyl guaiacol or 4-vinyl phenol from ferulic and *p*-coumaric acids (Figure 1), respectively, most are not able to reduce the vinyl intermediates to 4-ethyl guaiacol or 4-ethyl phenol (Chatonnet *et al.*, 1992; 1995; Shinohara *et al.*, 2000). Because of this observation, analysis of 4-ethyl phenol has been used as an indicator of *Brettanomyces* infections. However, some microorganisms, most notably *L. plantarum* (Chatonnet *et al.*, 1992; 1995; Cavin *et al.*, 1993) and *Pichia guilliermondii* (Dias *et al.*, 2003), are reported to produce either very small amounts of these ethyl phenols or do not survive in wine. The recent finding of isolating *Candida pararugosa* and *Pichia guilliermondii* from spoiled wines in Washington State not

containing *Brettanomyces* (data not shown) suggests that other microorganisms may, in fact, be able to produce volatile phenols in wines under certain conditions.

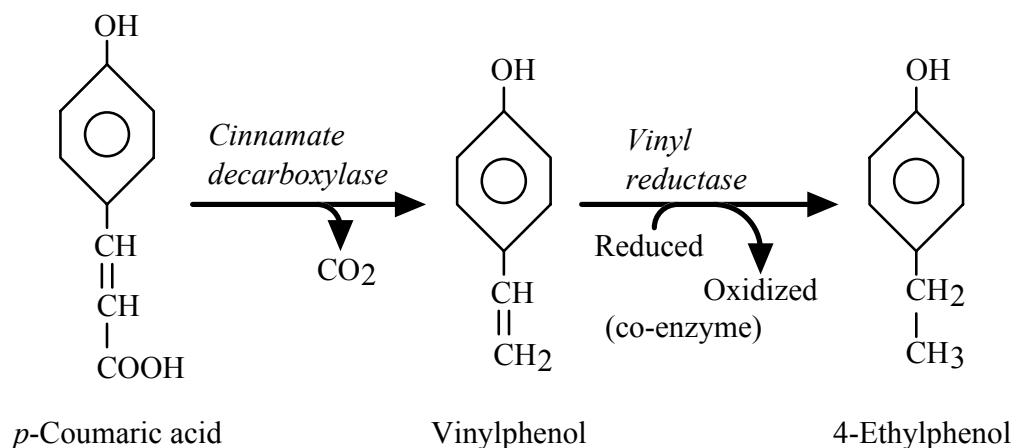


Figure 1. Formation of 4-ethyl phenol from *p*-coumaric acid.

Controlling the growth of the spoilage yeast within a winery is not an easy task. In fact, *Brettanomyces* appears to be relatively tolerant to sulfites so winemakers commonly add 0.4 to 0.6 mg/L molecular sulfur dioxide to limit infections. However, little information is available regarding the toxicity of sulfites towards this spoilage yeast.

### ***Pediococcus***

*Pediococci* are characterized as being spherical, Gram positive, non-motile, catalase negative, aerobic to microaerophilic microorganisms (Garvie, 1986; Carr *et al.*, 2002). *Pediococcus* is the only lactic acid bacterium that divide in two planes, thereby appearing microscopically as tetrads or large clumps of cells (Garvie, 1986; Axelsson, 1998). Currently approved species are *P. acidilacti*, *P. damnosus*, *P. dextrinicus*, *P. halophilus*, *P. inopinatus*, *P. parvulus*, *P. pentosaceus*, and *P. urinae-equi* (Garvie, 1986). The International Committee on Systematic Bacteriology has ruled that one species reported to be present in wines, *P. cerevisiae*, was not validly described as it represented at least two different species, *P. damnosus* and *P. pentosaceus* (Garvie, 1974; 1986; Raccach, 1987).

### Ecology

Although *P. damnosus* has been isolated from grape musts (Lonvaud-Funel *et al.*, 1991), little is known regarding the ecology of other species. In a comprehensive study by Costello *et al.* (1983), a number of lactic acid bacteria were isolated from musts and wines at different times during vinification including *Pediococcus* spp., in agreement with others (Lafon-Lafourcade *et al.*, 1983b; Fleet *et al.*, 1984; Davis *et al.* 1986a; 1986b; Sieiro *et al.*, 1990). Species of *Pediococcus* isolated from wine include *P. damnosus*, *P. pentosaceus*, *P. parvulus* and, to a lesser extent, *P. inopinatus* (Davis *et al.*, 1986b; Garvie, 1986; Edwards and Jensen, 1992; Manca de Nadra and Strasser de Saad, 1995). *Pediococci* are commonly found in red wines during barrel aging (Edwards and Jensen, 1992).

Besides pH, the growth of *Pediococcus* in wine is influenced by a variety of conditions including SO<sub>2</sub>, ethanol, and lysozyme. Edwards and Jensen (1992) reported that *pediococci* isolated from wines produced in Washington tended to grow slower in 30 mg/L total SO<sub>2</sub> and that only one of the ten strains analyzed grew in 14% ethanol. Work by Davis *et al.* (1988) with lactic acid bacteria isolated from

Australia red wines indicated that strains of *L. oenos* (*O. oeni*) were less tolerant to sulfur dioxide than strains of *P. parvulus*. Davis *et al.* (1988) further suggested that wines with high total SO<sub>2</sub> concentration may be more likely to support the growth of *Pediococcus* than *L. oenos*, in disagreement with Hood (1983) who reported that pediococci were less tolerant to bound SO<sub>2</sub> than lactobacilli or leuconostocs. *Pediococcus* spp. are also sensitive to lysozyme but more resistant than other bacteria. As an example, Delfini *et al.* (2004) noted that *P. parvulus* survived concentrations of 500 mg/L, generally higher than those of *Lactobacillus* (200 to 500 mg/L) or *Oenococcus* (50 to 100 mg/L).

#### Wine spoilage

*Pediococcus* spp. in wine has been generally considered to be undesirable due to the production of off-aromas and flavors like 'excessive butter', 'bitterness', or even 'dirty socks.' Pediococci are capable of producing diacetyl, a compound reminiscent of 'butter' that adversely affects wine quality at high concentrations (Sponholz, 1993). Some species are also capable of degrading glycerol to acrolein, a compound that reacts with phenolics to produce a bitter taint in wine (Davis *et al.*, 1988; Sponholz, 1993; Du Toit and Pretorius, 2000).

Besides producing off-flavors, *Pediococcus* spp. have been implicated in the production of extracellular polysaccharides. These homoglycans are produced from glucose and consist of a trisaccharide repeating unit having a (1→3)-linked backbone and a (1→2)-linked branch of one of the D-glucopyranosyl groups (Llauberes *et al.*, 1990). While visually unappealing, these polymers cause an increase in viscosity of the wine (Manca de Nadra and Strasser de Saad, 1995). Pediococci associated with this 'ropiness' defect are thought to have higher tolerances to ethanol than other strains (Du Toit and Pretorius, 2000).

*P. damnosus* is the bacterium primarily implicated in the production of polysaccharides in wine, although *P. pentosaceus* may also be involved (Manca de Nadra and Strasser de Saad, 1995; Lonvaud-Funel, 1999). As strains of *P. damnosus* that produce polysaccharides contain a unique 4 Kb plasmid, Lonvaud-Funel *et al.* (1993) developed a DNA probe for detection. More recently, Gindreau *et al.* (2001) reported use of a direct polymerase chain reaction (PCR) detection method to detect these strains.

Although growth of certain *Pediococcus* spp. in wines is undesirable, Edwards and Jensen (1992) isolated pediococci from several high quality commercial wines. Later work by Edwards *et al.* (1994) noted that some of these strains (*P. parvulus*) altered the bouquet of a Cabernet Sauvignon wine that had not undergone MLF but without spoilage. In addition, Silver and Leighton (1981) noted that strain B44-40, initially thought to be *Leuconostoc oenos* (*Oenococcus oeni*) but now believed to be *Pediococcus* (Kelly *et al.*, 1989), catalyzed MLF in wines without formation of off-odors or flavors. It is therefore possible that the growth of certain pediococci in wine may add desirable flavors and aromas under specific circumstances.

#### Interactions with *Oenococcus*

Because of the increase in pH after MLF, the secondary fermentation can encourage the growth of other lactic acid bacteria like *Pediococcus* in wine (Davis *et al.*, 1986a). However, Edwards *et al.* (1994) observed a definite antagonism by *O. oeni* against *Pediococcus* was noted in Cabernet Sauvignon and Merlot wines that had undergone MLF (Figure 2). Here, the viability of all strains of pediococci declined from an initial population of 10<sup>5</sup> to between <300 and 10<sup>4</sup> CFU/mL shortly after inoculation. For example, while the viability of WS-29A decreased ca 1 log, C5 could not be isolated in the MLF (+) Cabernet Sauvignon wine less than one hour after inoculation. The fact that the viability of *P. parvulus* decreased in MLF (+) wines after inoculation supports the contention that MLF can impart microbiological stability to wines. In agreement, Walling *et al.* (2005) noted that wines that had undergone MLF were more resistant to ropiness caused by pediococci. However, it is also clear that

the apparent biological “stability” imparted by MLF was not necessarily permanent because most strains of pediococci eventually grew to populations approaching or exceeding  $10^6$  CFU/mL.

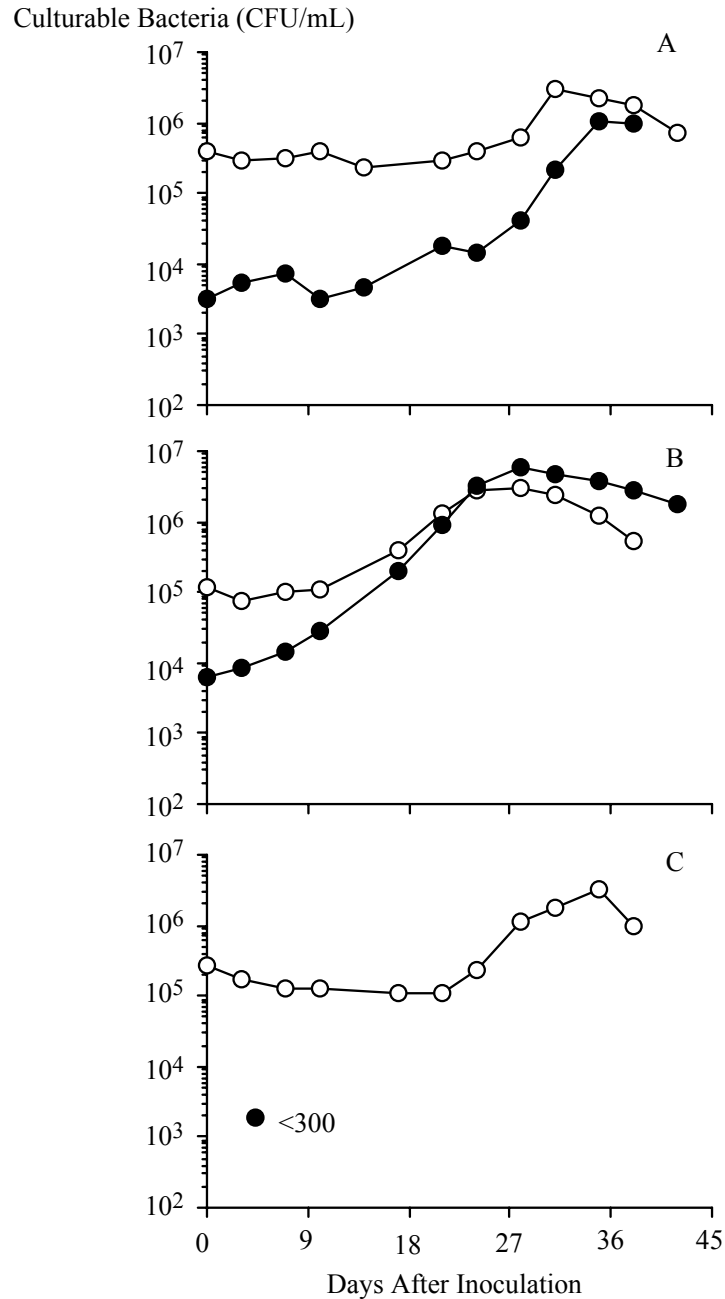


Figure 2. Growth of *Pediococcus parvulus* strain WS-9 (A), WS-29A (B), and C5 (C) inoculated into Merlot wines without MLF (open circles) or with MLF (closed circles) induced by *Oenococcus oeni*. Redrawn from Edwards *et al.* (1994).

Other reports detailing the complicated interactions between wine lactic acid bacteria have been published. Although *O. oeni* can inhibit *Pediococcus* (Edwards *et al.*, 1994), Davis *et al.* (1986a) noted that growth of *P. parvulus* was antagonistic to the survival of *O. oeni* after MLF in some red wines from

Australia. In addition, Lonvaud-Funel and Joyeux (1993) observed strong inhibition of *O. oeni* by a strain of *P. pentosaceus*. These authors theorized that the effect was due to accumulation of small (less than 1 kD) compounds, quite possibly peptides or proteins.

Besides other lactic acid bacteria, it is also possible that the growth of *Pediococcus* may impact other wine microorganisms such as *Brettanomyces*. It is well known that *Brettanomyces* produces 4-ethyl guaiacol and 4-ethyl phenol, compounds that originate from ferulic acid and *p*-coumaric acid, respectively. The reaction is a two step process with an initial decarboxylation of the hydroxycinnamic acids catalyzed by cinnamate decarboxylase and the reduction of the vinyl phenol intermediates by vinyl phenol reductase (Figure 1). While many wine microorganisms like *Acetobacter*, *O. oeni*, *L. hilgardii*, *L. plantarum*, *L. brevis*, *P. pentosaceus*, *P. damnosus*, and *Saccharomyces* can synthesize 4-vinyl guaiacol or 4-vinyl phenol from ferulic and *p*-coumaric acids, respectively, most are not able to reduce the vinyl intermediates to 4-ethyl guaiacol or 4-ethyl phenol (Chatonnet *et al.*, 1992; 1995; Shinohara *et al.*, 2000; Dias *et al.*, 2003).

Although the specific co-enzyme involved in the second reaction has not been identified, one possible benefit to *Brettanomyces* would be reoxidation of NADH. Under anaerobic conditions such as those found in wines, the availability of NAD<sup>+</sup> can be limited such that carbohydrate metabolism is inhibited. As such, *Brettanomyces* could theoretically reduce vinyl phenols synthesized by other wine microorganisms, and thereby benefit from the growth of other microbes during aging (e.g., *Pediococcus*).

### **Lactobacillus**

Species of *Lactobacillus* isolated from grapes and wines worldwide include *L. brevis*, *L. buchneri*, *L. casei*, *L. cellobiosus*, *L. curvatus*, *L. delbrueckii*, *L. hilgardii*, *L. jensenii*, *L. kunkeei*, *L. leichmanni*, *L. nagelii*, *L. plantarum*, and *L. trichodes* (Douglas and Cruess, 1936; Vaughn, 1955; Fornachon, 1957; Du Plessis and van Zyl, 1963; Pilone *et al.*, 1966; Chalfan *et al.*, 1977; Maret and Sozzi, 1977; 1979; Costello *et al.*, 1983; Lafon-Lafourcade *et al.*, 1983b; Davis *et al.*, 1986a; 1986b; Dicks and van Vuuren, 1988; Sieiro *et al.*, 1990; Edwards *et al.*, 1993; 1998a; 2000). *L. cellobiosus* is regarded as a biotype of *L. fermentum* while *L. leichmanni* is now referred to as *L. delbrueckii* subsp. *lactis* (Kandler and Weiss, 1986). *L. trichodes*, commonly known as "Fresno mold" (Amerine and Kunkee, 1968), is considered a synonym of *L. fructivorans* (Weiss *et al.*, 1983). *L. vermiforme*, a species also found in wines (unpublished data), is believed to be incorrectly classified and should belong to *L. hilgardii*. The actual relationship between *L. vermiforme* and *L. hilgardii* has not been resolved.

While *Lactobacillus* can induce the secondary fermentation of wines, the malolactic fermentation, most species are considered to be spoilage microorganisms (Vaughn, 1955; Gini and Vaughn, 1962; Lafon-Lafourcade *et al.*, 1983a; Davis *et al.*, 1985; 1986b; Wibowo *et al.*, 1985). For instance, some lactobacilli can produce diacetyl and acetoin (El-Gendy *et al.*, 1983; Benito de Cárdenas *et al.*, 1985; Montville *et al.*, 1987) or substituted tetrahydropyridines (Heresztyn, 1986), compounds which can impart undesirable characteristics to wines. Growth of lactic acid bacteria in bottled wines can result in haze formation, sediment, gassiness, off-odors, excessive volatile acidity and/or lactic acid (Rankine and Bridson, 1971; Splittstoesser and Stoyla, 1987). Although lactobacilli can not generally grow at alcohol concentrations >15% (v/v) or at pH levels less than 3.5 (Wibowo *et al.*, 1985), strains of *L. trichodes* have been isolated from spoiled dessert wines containing as much as 20% alcohol (Splittstoesser and Stoyla, 1987). Furthermore, *L. plantarum* can also tolerate pH levels below 3.5 (Edwards *et al.*, 1993).

### Stuck alcoholic fermentations

A serious problem encountered sporadically by a winemaker are sluggish or stuck alcoholic fermentations. In such a wine, there is premature cessation of yeast growth and alcoholic fermentation

which produces a wine with unfermented sugars and an ethanol concentration lower than expected (Fleet and Heard, 1993). From a commercial standpoint, sluggish or stuck wines are a problem due to their sweeter taste, inferior sensory quality, and the potential for bacterial spoilage. Although various factors can contribute to a sluggish or stuck alcoholic fermentation, the exact cause(s) of a particular occurrence can not always be identified. Traditionally, factors shown to produce this problem are nutritional deficiencies, the presence of inhibitory substances, and technological practices.

More recently, some winemakers have observed rapid wine spoilage by microorganisms dubbed the "ferocious" lactobacilli (Boulton *et al.*, 1996). Boulton *et al.* (1996) characterized this spoilage as being very swift with abundant bacterial growth during the early stages of vinification and a premature stoppage of alcoholic fermentation. Although interactions between yeasts and lactic acid bacteria in wines have been extensively studied (Beelman *et al.*, 1982; King and Beelman, 1986; Lemaesquier, 1987; Edwards *et al.*, 1990; Cannon and Pilone, 1993), the first confirmed report that *Lactobacillus* can actually inhibit yeast was that of Huang *et al.* (1996). In their study, early inoculation of strains YH-15, YH-24, and YH-37 resulted in slowed laboratory-scale Chardonnay fermentations catalyzed by *Saccharomyces cerevisiae* Epernay 2. Inoculation of YH-15 also slowed fermentations inoculated with *S. cerevisiae* EC1118. The rapid growth of YH-15 in unsulfited grape juice observed by Huang *et al.* (1996) fit the description of "ferocious" lactobacilli given by Boulton *et al.* (1996) in that the strain achieved a peak population of  $>10^9$  CFU/mL two days after inoculation. Inhibition of yeast by *Lactobacillus* had been previously observed in other foods (Nakamura and Hartman, 1961; Noda *et al.*, 1980; Barbour and Priest, 1988; Essia Ngang *et al.*, 1990; Leroi and Pidoux, 1993).

Using 16S rRNA sequencing techniques, Edwards *et al.* (1998a) identified strain YH-15 as a novel species of *Lactobacillus* and named the strain *Lb. kunkeei*. Interestingly, strains YH-24 and YH-37 also isolated by Huang *et al.* (1996) were later determined to be wild types of *O. oeni* (Edwards *et al.*, 1998b). More recently, another strain of *Lactobacillus* was found to inhibit yeast based on results from an agar well assay. This strain, LuE<sub>10</sub>, has been identified as another novel species and was named *Lb. nagelii* (Edwards *et al.*, 2000).

#### Mechanism of yeast inhibition

Boulton *et al.* (1996) indicated that enough acetic acid could be produced by "ferocious" lactobacilli in two or three days to inhibit yeast metabolism. Acetic acid is well known to be inhibitory to yeasts (Doores, 1993), influencing both growth and fermentative abilities (Pampulha and Loureiro, 1989; Ramos and Madeira-Lopes, 1990; Kalathenos *et al.*, 1995). In agreement, Rasmussen *et al.* (1995) reported that addition of 4 g/L midway through grape juice fermentations slowed the fermentations.

Huang *et al.* (1996) reported that slow/stuck fermentations inoculated with *L. kunkeei* contained high amounts of volatile acidity, approximately 3 g/L. In agreement, Edwards *et al.* (1999b) noted that the acetic acid concentration in musts inoculated with *L. kunkeei* or with both *S. cerevisiae* and *L. kunkeei* approached 5 g/L. Thus, the high volatile acidities present in wines inoculated with *L. kunkeei* observed by Huang *et al.* (1996) were probably a result of bacterial production of acetic acid rather than being synthesized by yeast. However, it was not known whether this amount of acetic acid would inhibit yeast growth and induce slow/stuck fermentations. Later work revealed that sequential addition of acetic acid to a fermenting must in the same concentrations produced by *L. kunkeei* resulted in a slower alcoholic fermentations (Edwards *et al.*, 1999b). This finding supported the concept that bacterial production of acetic acid can slow alcoholic fermentations but other mechanisms are probable.

From a winemaking standpoint, it should be noted that SO<sub>2</sub> remains the best method to control *Lactobacillus* infections (Hood, 1983; Liu and Gallander, 1983; Wibowo *et al.*, 1985; Edwards *et al.*, 1999a). Like other wine microorganisms, lactic acid bacteria possess varying tolerances to the additive (Beelman *et al.*, 1977; Wibowo *et al.*, 1985; Davis *et al.* 1986a; 1986b; 1988; Britz and Tracey, 1990;

Edwards *et al.*, 1991; 1993). Bound or free SO<sub>2</sub> is not highly inhibitory to wine microorganisms; rather, it is the molecular form of SO<sub>2</sub> that is antimicrobial. The amount of molecular SO<sub>2</sub> present in a wine is dependant on the concentration of free SO<sub>2</sub> and the must/wine pH (Zoecklein *et al.*, 1995). As a general, 0.8 mg/L molecular SO<sub>2</sub> is considered to be completely inhibitory to lactic acid bacteria (Zoecklein *et al.*, 1995). Although lactobacilli and pediococci can be thought of as being more sensitive to SO<sub>2</sub> than *O. oeni*, some studies (Costello *et al.*, 1983; Davis *et al.*, 1986a; 1988; Edwards *et al.*, 1993) have observed growth of species of these microorganisms in wines containing >30 mg/L total SO<sub>2</sub>. Fortunately, it appears that some of the lactobacilli implicated in sluggish fermentations are very sensitive to SO<sub>2</sub> (Edwards *et al.*, 1999a).

### Detection Methods

Methods used for detection of microbial spoilage include both chemical (e.g., volatile acidity, 4-ethyl phenol, biogenic amines, etc.) and microbiological (e.g., plating on specific media, biochemical methods, etc.). Perhaps one of the more important methods applied in wineries has been the use of a phase contrast microscope. Here, the microscopic appearance of individual cells is used for preliminary identification using established guides (Edwards, 2005). Although prone to human error in recognizing cell morphologies, this method is rapid and relatively inexpensive.

In an effort to improve accuracy and precision, techniques involving qualitative and quantitative analysis of DNA are also being utilized (Fugelsang and Edwards, 2007). In general, these methods rely on primers which react with selected portions of DNA from microorganisms like the Scorpions™ technique (Arvik, 2006). Besides detection of specific microorganisms, these methods have been used to quantify the number of microbes present (more DNA in the wine = higher population of microorganisms). However, detection and quantitation relies on the assumption that DNA from microorganisms remains detectable in a wine for only a short period of time, an assumption that has been questioned (Mills, 2006). Furthermore, it is important that these methods be able to detect all microbial species known to be present in wines (not all methods have the necessary primers).

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