Understanding Problem Fermentations – A Review

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Despite advances in winemaking technology and improvements in fermentation control, problem alcoholic and malolactic fermentations remain a major oenological concern worldwide. This is due to possible depreciation of product quality and its consequent negative economic impact. Various factors have been identified and studied over the years, yet the occurrence of fermentation problems persists. The synergistic effect of the various factors amongst each other provides additional challenges for the study of such fermentations. This literature review summarises the most frequently studied causes of problematic alcoholic and malolactic fermentations and in addition provides a summary of established and some potential new analytical technologies to monitor and investigate the phenomenon of stuck and sluggish fermentations.

INTRODUCTION

Alcoholic fermentation, the conversion of the principal grape sugars, glucose and fructose, to ethanol and carbon dioxide is conducted by yeasts of the genus *Saccharomyces*, generally *S. cerevisiae* and *S. bayanus* (Boulton *et al.*, 1996). This complex microbial process probably represents the oldest form of biotechnological applications of a microorganism and has been used by humans for several thousand years (Samuel, 1996).

Despite considerable improvements in our ability to monitor and control fermentation, stuck and sluggish fermentations remain major challenges for the international wine industry, including South Africa. Bisson (1999) defined incomplete or "stuck" fermentations as those having a higher than desired residual sugar content at the end of alcoholic fermentation, while slow or "sluggish" fermentations are characterised by a low rate of sugar consumption by the yeast. Different types of problem fermentations are shown in Fig. 1 (discussed in Bisson, 2005). The economic and logistical consequences of sluggish and stuck wine fermentations in industrial cellars demand significant investigation into the causes and the determination of methods to avoid this problem.

One of the earliest reports relating to problematic or abnormal fermentations date back to the late 1800's with the pioneering work of Louis Pasteur (reviewed by Barnett, 2000). During this period Pasteur demonstrated that the desired production of alcohol in fermentation is due to yeast and that the undesired production of substances (such as lactic acid and acetic acid) which made the wine sour was due to the presence of additional organisms such as bacteria. Extensive research has been conducted since 1977 on elucidating problem fermentations and several causes of sluggish and stuck fermentation have been identified (Ingledew and Kunkee, 1985; Allen and Auld, 1988; Fugelsang *et al*., 1991; Kunkee, 1991; Bisson, 1993; Henschke and Jiranek, 1993; Henschke, 1997; Alexandre and Charpentier, 1998; Bisson, 1999). Factors such as high initial sugar content (Lafon-Lafourcade *et al*., 1979), nitrogen deficiency (Agenbach, 1977; Ingledew and Kunkee, 1985; Bely *et al*., 1990), vitamin deficiency, especially thiamine (Peynaud and Lafourcade, 1977; Ough *et al.*, 1989; Salmon, 1989), oxygen deficiency (Thomas *et al.*, 1978; Traverso Rueda and Kunkee, 1982), excessive must clarification (Groat and Ough, 1978; Houtman and Du Plessis, 1986; Alexandre *et al*., 1994), high ethanol concentrations (Casey and Ingledew, 1986), inhibition of yeast cell activity by fermentation by-products, particularly the fatty acids (Geneix *et al*., 1983; Lafon-Lafourcade *et al*., 1984; Viegas *et al*., 1989; Edwards *et al*., 1990) and acetic acid (Kreger-Van Rij, 1984; Edwards *et al.*, 1999), pH (Kado *et al.*, 1998), killer toxins (Barre, 1982; Van Vuuren and Jacobs, 1992), and pesticides (Doignon and Rozes, 1992) have all been identified as potentially responsible for fermentation problems. In addition to the individual effects of each of these factors, possible synergistic effects amongst them add to the complexity of understanding problem fermentations. For this reason the prediction and diagnosis of the exact causes of problem fermentations are often rendered extremely challenging.

Similar to alcoholic fermentation, stuck and sluggish malolactic fermentations are also frequently occurring and difficult to manage in the wine industry. Malolactic fermentation is the secondary fermentation during the winemaking process and especially important for the ageing of red wines and certain white wines (Fugelsang, 1996; Du Plessis, 2005). During this process, catalysed by lactic acid bacteria, malic acid is converted to lactic acid. Factors influencing the successful onset and completion of malolactic fermentation include ethanol content, pH, SO_2 levels, temperature, nutritional requirements and microbial incompatibility (reviewed by Davis *et al.*, 1985, 1986, 1988).

In this literature review, the causative factors of problem fermentations and general factors influencing fermentation efficiency will be discussed in more detail for both alcoholic and malolactic fermentation. These will include physical (pH and temperature), chemical (nutrients and inhibitory substances) and microbiological factors (microbial competition) and the potential synergistic

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effects amongst these factors. The issue of must composition, especially the nitrogen content and glucose:fructose ratio, has a definite impact on fermentation efficiency. Since the must composition is also dependent on viticultural practices and harvest considerations, these factors will also be discussed. Along with the development of analytical technology and increased availability of statistical techniques (chemometrics), potentially new and alternative techniques to monitor fermentation evolved. The last section of this review will highlight a selection of these analytical methods and chemometric applications, which could potentially be used to effectively monitor fermentation progress.

CAUSES OF FERMENTATION ARREST

A spectrum of possible factors, from the vineyard to the cellar, will be discussed in this section. The layout of these sections are summarised in Table 1.

Vineyard and viticultural factors

Fermentation problems can already originate from the vineyard as the must composition influences the fermentation efficiency. The concentration of nitrogen and yeast-required micronutrients is influenced by a variety of parameters. These include grapevine nutrient deficiencies, fungal degradation and degree of fruit maturity at harvest which is predetermined by cultivar, rootstock, crop load, canopy management, vineyard fertilization and climate (Kliewer, 1970).

Vineyard nitrogen fertilization influences the concentrations of nitrogenous compounds in juice (Spayd *et al.*, 1991, 1994). This affects the formation of higher alcohols and esters by yeast during fermentation (Ough and Bell, 1980; Ough and Lee, 1981; Gallander *et al*., 1989; Webster *et al*., 1993) and therefore indirectly wine quality. Spayd *et al*. (1994) found that an increased rate of nitrogen fertilization resulted in increased concentrations of all nitrogen fractions, including individual amino acids, in White Riesling juice. Nitrogen fertilization increased Merlot must arginine concentrations from 279 to 798 mg/L and proline from 1062 to 1639 mg/L in a Bordeaux study (Bertrand *et al*., 1991). Nitrogen deficiencies in juice can limit yeast growth (Agenbach, 1977; Salmon, 1989; Monteiro and Bisson, 1991; Reed and Nagodawithana, 1991; Spayd *et al*., 1991) therefore resulting in sluggish or stuck fermentations (Agenbach, 1977; Vos, 1981; Salmon, 1989; Kunkee, 1991; Spayd *et al*., 1991) and in the release of H_2S (Vos and Gray, 1979; Henschke and Jiranek, 1991; Jiranek, 1995a).

Agricultural residues (pesticides, fungicides, herbicides) on the exterior surface of grape fruit could also influence fermentation performance (further discussed in the section on inhibitory substances).

Various cultivars exhibit different glucose and fructose levels in their berries (Kliewer, 1965; Snyman, 2006). The glucose:fructose ratio changes from season to season as a result of climate and ripeness level. Snyman (2006) reported increased fructose levels (lower glucose:fructose ratio) in the grapes of different cultivars during warm and dry seasons. This corresponds to the results obtained by Kliewer (1965). Theories to explain this phenomenon include the conversion of glucose to fructose with sorbitol as an intermediate product. It is not clear whether this reaction is enhanced by increased temperature and/or whether a closer link with other complex biochemical processes exist (Snyman, 2006). Another theory involves the degradation of glucose in the grape berry by the pentose phosphate cycle. If sucrose enters the berry and glucose is metabolised, the fructose levels would increase and the ratio of glucose:fructose would be reduced. In the case of overripe grapes, increased time on the vine would re-

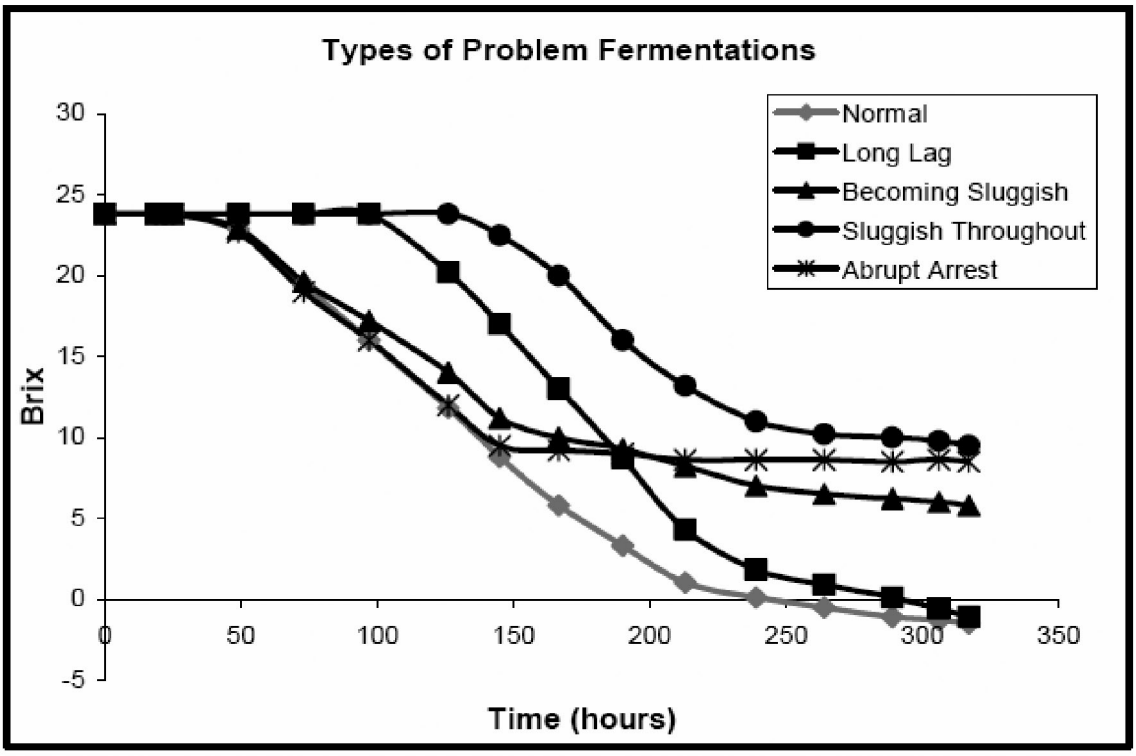

FIGURE 1
Types of problem fermentations observed in Californian production conditions (Bisson, 2005).

TABLE 1

The causes of fermentation arrest can originate from multiple factors some of which are already present in the vineyard. This section is therefore divided into various subsections explaining in each section where fermentation problems could originate from.

Causes of Fermentation Arrest
Factors
Vineyard and viticultural
Harvest conditions
Cellar management: alcoholic fermentation
Cellar management: malolactic fermentation

sult in more glucose degradation through respiration leading to a reduced glucose:fructose ratio. The majority of biochemical reactions occur faster during warmer seasons and this could explain the lower levels of glucose in relation to fructose present in the berry (Snyman, 2006). Viticulturally, overripeness can be avoided by monitoring the glucose:fructose ratio during ripening to avoid consequent fermentation problems. The aspects related to glucose:fructose ratios and harvest will be discussed in more detail in the following section on harvest conditions.

Harvest conditions

The rate of fermentation by yeast and bacteria is considerably influenced by the amino acid composition of the must. It has been reported that the fruit proline concentrations increase as the °Brix increase during ripening (Ough, 1968; Kliewer, 1970). The increase or decrease of arginine with increased fruit maturity is reported as dependent on the specific cultivar (Kliewer, 1970). Changes in the amino acid profile of grapes during the ripening process have been studied extensively (Kliewer, 1968, 1970; Huang and Ough, 1991; Lehtonen, 1996; Spayd and Andersen-Bagge, 1996; Hernández-Orte *et al*., 1999; Nicolini *et al*., 2001) and a wide range of free amino nitrogen concentrations at harvest maturity has been reported (Vos, 1981), depending on the region, cultivar and growing conditions of the grapevine. Peynaud and Lafon-Lafourcade (1961) reported an increase in the less assimilable nitrogen forms (proline and threonine) as grapes ripen. This could explain why musts of overripe grapes sometimes ferment slowly (Kliewer, 1968).

Climatic changes each year often result in various vineyards in a specific viticultural region achieving optimal ripeness simultaneously. This puts enormous pressure on cellars to process these grapes and could result in the pressing of certain vineyards at higher sugar levels and increased grape maturity than desired. In addition to the influence of grape nitrogen content on fermentation, the glucose and fructose concentrations in grapes also exhibit a tremendous effect on fermentation performance. The subject of glucose and fructose concentrations in grapes has been extensively investigated over the years (Amerine, 1954; Amerine and Thoukis, 1958; Kliewer, 1965, 1968; Snyman, 2006). It was found that glucose predominates in unripe grapes, the glucose:fructose ratio at fruit maturity is about 1 and that fructose constitutes the major sugar in overripe grapes. Kliewer (1965) reported a sudden decrease in the glucose:fructose ratio as fruit becomes over matured and Snyman (2006) reported similar results. These results indicate that over mature grapes become increasingly detrimental for successful fermentation unless the correct yeast strain is used or a different wine style is desired.

Vineyard mechanization includes mechanical leaf removal, pruning, fruit thinning and harvesting and is a reality of modern viticultural technology (Morris, 2000). The major quality problem associated with machine harvested grapes is the fruit damage and the handling after harvest (Moyer *et al*., 1961; Shepardson and Miller, 1962; Bourne *et al*., 1963; Marshall *et al*., 1971; Christensen *et al*., 1973). However, with the development of technology, the harvesting machine improved with regard to less fruit damage (Morris, 2000). It should still be kept in mind that a considerable interval between machine harvesting and processing of the grapes can result in increased enzymatic activity and browning, oxidation (loss of color) and development of off-flavours and microbial growth (Bourne *et al*., 1963; Marshall *et al*., 1971; Marshall *et al*., 1972; Christensen *et al*., 1973; Splittstoesser *et al.* 1974; Peterson, 1979). Temperature during this time interval influences the quality of machine harvested grapes tremendously (Marshall *et al.*, 1971; 1972; Morris *et al.*, 1972, 1973, 1979; Peterson, 1979). The transport of machine harvested grapes from the vineyard to wineries could enhance the onset of alcoholic fermentation (of the released juice) by wild yeasts. The resulting high initial wild yeast populations could produce high concentrations of acetic acid and ethanol resulting in inhibition of the desired yeast starter culture or fermentation difficulties (Morris *et al.*, 1973; Alexandre and Charpentier, 1998). Sulphur dioxide addition to machine harvested grapes has been shown to discourage bacterial spoilage and can serve as an antioxidant to prevent juice browning (Bourne *et al.*, 1963; Morris *et al.*, 1972, 1973, 1979; Nelson and Ahmedullah, 1972; Benedict *et al.*, 1973; Christensen, 1973; O'Brien and Studer, 1977). The above-mentioned considerations and precautions are also applicable for hand harvested grapes, however, due to the increased fruit damage observed for machine harvested grapes, the effect might be more detrimental to yeast fermentation.

Cellar management: alcoholic fermentation

This section discusses factors in the cellar which could influence alcoholic fermentation. A summary of the different sections is given in Table 2.

Yeast strain

Yeast performance is determined partly by its genetic makeup (genotype), which is species and strain dependent. Strain differences are more pronounced in stress conditions, suggesting differences in adaptation to the environment, a hypothesis that is supported by transcriptome data (Gasch, 2003). Wine yeast strains differ largely in nitrogen requirements and ability to utilise sugars, especially during the later stages of fermentation (McClellan *et al*., 1989; Schütz and Gafner, 1995). Selection of yeast strains which efficiently utilise available nitrogen in low nitrogen musts and juices, in addition to nitrogen supplementation appears to be one approach to resolve fermentation difficulties due to nitrogen deficiencies (Jiranek *et al*., 1991, 1995b). Strains also differ in their ability to utilise glucose (glucophilic yeast) and fructose (fructophilic yeast). The selection of appropriate fructophilic yeasts for fermentations of grapes suspected to have low glucose:fructose ratios could avoid fermentation problems.

TABLE 2

Factors in the cellar environment that could influence alcoholic fermentation.

Cellar Management: Alcoholic Fermentation	
Sections	**Subsections**
Yeast strain	
Yeast preparation	
Yeast nutrition	Nitrogen
	Phosphate
	Oxygen and other survival factors
	Vitamins
	Minerals
Inhibitory substances	Ethanol
	Acetic acid
	Medium chain fatty acids
	Toxins and killer toxins
	Sulphites
	Agricultural residues
Physical factors	Excessive must clarification
	pH
	Temperature extremes
Microbial incompatibility	
Metabolic basis of stuck and sluggish fermentations	

Challenging fermentation conditions such as high level of juice clarification, high protection from air (low oxygen content), low assimilable nitrogen and high sugar content requires yeast strains to have a high sugar and ethanol tolerance to complete fermentation successfully without producing any off-flavours (Henschke, 1997). Degré (1993) described various characteristics for the selection of good wine yeast strains to conduct fermentation successfully. Tolerance to both ethanol and temperature is also very strain dependent (Bisson, 1999).

Yeast preparation

Apart from the importance of yeast strain selection, the preparation of the inoculum is equally critical. In order to achieve maximum viability, commercial active dried yeast should not be directly inoculated into the must. Rehydration, according to the manufacturer's instructions, at the recommended temperature without exceeding the recommended rehydration period is required to re-establish functional membranes and metabolic activity (Boulton *et al*., 1996). The suspension should be mixed properly, although excessive mixing could result in loss of cell viability (Bisson, 2005). Deviations from the rehydration instructions such as extended rehydration in water and cold or hot rehydration will reduce the yeast viability (Bisson, 2005). During rehydration and inoculation the yeast is exposed to respectively hypo-osmotic and hyper-osmotic shock (Bauer and Pretorius, 2000). Additional temperature shock (5 to 7°C difference between culture and must temperature) when rehydrated yeast is introduced into the must greatly reduces the cell concentration of the inoculum (Zoecklein, 2005). Ingledew and Kunkee (1985) showed high cell numbers promoted faster rates of fermentation. The use of old or expired active dried yeasts might also cause fermentation problems.

Initial yeast populations should be large enough ($2x10^6$ to $5x10^6$ yeast cells/mL) (Zoecklein, 2005) to dominate indigenous microflora and ensure rapid, complete fermentation (Bauer and Pretorius, 2000). Unsuccessful inoculation could result in incomplete fermentation due to the growth of less alcohol tolerant indigenous yeast (Henschke, 1997).

Yeast nutrition

Nitrogen – essential macronutrients

Nitrogenous compounds are important components of grape juice and impact on the production of yeast biomass, fermentation rate and time to complete fermentation (Bisson, 1991). The formation of fermentation flavours, such as hydrogen sulphide, organic acids (excluding tartaric acid), higher alcohols and esters are also influenced by nitrogen (Bell *et al.*, 1979; Simpson, 1979; Vos and Gray, 1979; Ough and Bell, 1980; Vos, 1981; Juhasz and Torley, 1985; Dukes *et al*., 1991; Henschke and Jiranek, 1991; Rapp and Versini, 1991; Jiranek *et al*., 1995a; Webster *et al.*, 1993). This spectrum of yeast metabolism end products directly influences wine quality.

Saccharomyces yeast species are capable of synthesizing all required nitrogen-containing compounds from ammonium (NH_4^+), carbon and energy sources. Ammonia and free alpha amino acids (collectively referred to as FAN) are therefore readily assimilated, while peptides and proteins are assimilated for the production of amino acids via hydrolysis (Reed and Nagodawithana, 1991). Nitrogenous compounds are used by yeast to produce structural and functional proteins that result in increased yeast biomass and the production of enzymes that facilitate many biochemical changes occurring during yeast fermentation (Spayd and Andersen-Bagge, 1996). The importance of nitrogenous compounds in fermentation of grape juice and beer worts were reviewed by Bisson (1991) and O'Connor-Cox and Ingledew (1989), respectively.

Nitrogen deficiency (less than 150 mg/L FAN) slows down yeast growth and the fermentation or may even result in a stuck fermentation (Agenbach, 1977; Vos *et al*., 1978; Monk, 1982; Jiranek *et al*., 1991; Kunkee, 1991; Monteiro and Bisson, 1991; Butzke and Dukes, 1996), possibly due to the inhibition of the synthesis of proteins transporting sugar through the cell membrane to the interior of the cells (Busturia and Lagunas, 1986; Salmon, 1989; Huang and Ough, 1991). It has been shown that an adequate supply of nitrogen increases yeast growth provided the other essential yeast nutrients are not lacking (Aries and Kirsop, 1977; Strydom *et al*., 1982; Ingledew and Kunkee, 1985; Henschke, 1990; Dukes *et al*., 1991). However, additions of ammonia after the early yeast growth phase may be ineffective in that the inhibited sugar transport into yeast cells may be irreversible in low nitrogen juices (Salmon, 1989). Yeast may use amino acids not only as nitrogen sources but also as redox agents to balance the oxidation-reduction potential under conditions of restricted oxygen (Albers *et al*., 1996; Mauricio *et al.*, 2001).

Phosphate

Phosphate limitation has been shown to affect cell growth and biomass formation as well as directly affecting fermentation rate (Lafon-Lafourcade and Ribéreau-Gayon, 1984; Gancedo and Serrano, 1989; Boulton *et al*., 1996).

Oxygen and other survival factors

Oxygen and/or the presence of certain lipids, referred to as oxygen substitutes, are critical for yeast growth (Munoz and Ingledew, 1989a, 1989b, 1990). These 'survival factors' are compounds

that decrease the inhibitory effects of ethanol (Lafon-Lafourcade *et al*., 1979; Lafon-Lafourcade and Ribéreau-Gayon, 1984).

During the early stages of growth, the oxygen consumed by yeast appears to have an energy role (Henschke, 1997). Oxygen is essential for the biosynthesis of sterols and unsaturated fatty acids which are both essential to membrane structure and function (Casey and Ingledew, 1986) and cell viability.

The production of toxic fatty acids, octanoic and decanoic acid, are affected by oxygen deprivation (Bardi *et al.*, 1999) and the toxicity of these medium chain fatty acids (MCFA) increases as the ethanol concentration increases (Henschke, 1997). This effect elevates the risk of problem fermentations to occur.

Oxygen deficiency could be responsible for sluggish fermentation as a consequence of inhibition of lipid biosynthesis which results in decreased ergosterol and unsaturated fatty acid content ('survival factors'), decreased biomass production and yeast viability.

Must aeration could therefore stimulate lipid biosynthesis, increase ethanol tolerance as a result of increased lipid composition in the cell membrane, decrease the release of MCFA and reduce the potential toxicity and the risk of fermentation problems (Henschke, 1997; Alexandre and Charpentier, 1998).

The addition of yeast hulls, the cell wall material remaining after yeast extract preparation, has been suggested as supplements to juice to prevent stuck fermentations (Ribereau-Gayon, 1985). Studies showed the ability of yeast hulls to remove certain toxic fermentation side-products (Lafon-Lafourcade, 1984). In addition, Munoz and Ingledew (1989b) reported that yeast hulls could also supply beneficial unsaturated fatty acids and the importance of yeast hulls in the stimulation of fermentation and prevention of stuck and sluggish fermentations was verified (Munoz and Ingledew,1989a).

Vitamins

Insufficient availability of vitamins (essentially thiamine) has been associated with sluggish fermentations (Peynaud and Lafon-Lafourcade, 1977; Ough *et al*., 1989). *Saccharomyces cerevisiae* is capable of synthesising all essential vitamins except biotin, however, research has shown the presence of extracellular vitamins is highly stimulatory to growth and fermentation (Monk, 1982; Lafon-Lafourcade and Ribéreau-Gayon, 1984; Ough *et al.*, 1989; Fleet and Heard, 1993). It was shown that wild yeasts, such as *Kloeckera apiculata*, decrease thiamine levels to a deficient situation for *Saccharomyces* (Bataillon *et al.*, 1996). Acetic acid has been reported to reduce the ability of *Saccharomyces* to transport and retain thiamine (Iwashima *et al*., 1973). Thiamine is cleaved and its biological activity destroyed by sulphur dioxide, further reducing the concentration of this vitamin (Alexandre and Charpentier, 1998; Bisson, 1999).

Minerals

Deficiencies and imbalances in minerals and cations, serving as co-factors for glycolytic and other enzymatic reactions, can result in fermentation arrest (Dombeck and Ingram, 1986; Blackwell *et al.*, 1997; Walker and Maynard, 1997). Magnesium plays a key role in metabolic control, growth and cell proliferation, glycolytic pathway and subsequently ethanol production (reviewed by Walker, 1994). Limitation of zinc and magnesium directly affects sugar catabolism and consequently fermentative activity (Jones *et al*., 1981; Jones and Greenfield, 1984; Dombek and Ingram, 1986; D'Amore *et al.*, 1987; Monk, 1994). Calcium limitation increases ethanol sensitivity (Nabais *et al*., 1988). High manganese depresses uptake of magnesium and vice versa (Blackwell *et al*., 1997) which may lead to a deficiency situation. Additionally, an imbalance of pH and potassium ions present in grapes from vines with poor potassium uptake ability from the soil could result in stuck fermentations (Kudo *et al.*, 1998).

Inhibitory substances

Ethanol

Ethanol inhibits different transport systems utilised by *S. cerevisiae* (Leao and Van Uden, 1982; Cartwright *et al*., 1987b; Pascual *et al.*, 1988; Mauricio and Salmon, 1992; Salmon *et al*., 1993), influences proton fluxes (Leao and Van Uden, 1984; Cartwright *et al*., 1986, Cartwright *et al*., 1987a; Killian *et al*., 1989) and affects yeast plasma membrane composition (Jones and Greenfield, 1987; Jones, 1989, 1990) resulting in subsequent growth inhibition (Thomas and Rose, 1979; Ingram and Butke, 1984) and decrease in fermentation rate as a result of inhibiting sugar transport activity (Salmon *et al*., 1993). Fermentation temperature influences ethanol tolerance. At lower temperatures, greater tolerance to ethanol occurs (Henschke, 1997). An important property of ethanol is that it increases the toxicity of other compounds. The availability of sterols and fatty acids has a definite impact on ethanol sensitivity (Lafon-Lafourcade and Ribéreau-Gayon, 1984).

Acetic acid

High levels of acetic acid are often associated with stuck or sluggish fermentations. The heterofermentative lactic acid bacteria, including strains of *Lactobacillus* and *Oenococcus*, certain non-*Saccharomyces* yeasts such as *Brettanomyces* spp., *Hansenula anomala*, *Kloeckera apiculata* and *Candida krusei* (Fleet and Heard, 1993), commercial wine yeasts (Hanneman, 1985) and acetic acid bacteria (Drysdale and Fleet, 1985, 1988, 1989) all have the ability to produce high levels of acetic acid that directly increases volatile acidity (Lambrechts, 2000). Elevated acetic acid concentrations can inhibit yeast growth, enhance ethanol toxicity and prevent the completion of fermentation. Contrariwise, the arrest of fermentation could allow the growth of spoilage organisms which could lead to high levels of volatile acidity.

Medium chain fatty acids

Medium chain fatty acids which are intermediates in the biosynthesis of long chain fatty acids can inhibit alcoholic fermentation (Lafon-Lafourcade *et al*., 1984). Fatty acid toxicity increases as pH decreases with decanoic acid being more inhibitory than octanoic acid (Viegas *et al.*, 1989). Both inhibit hexose transporter systems resulting in reduced fermentation rate (Zamora *et al.*, 1996).

Toxins and killer toxins

Killer yeast strains (phenotype K^+R^+) secrete a proteinaceous extracellular toxin that kills other sensitive yeast strains (phenotype K^-R^-) of *S. cerevisiae*. Neutral yeasts (phenotype K^-R^+) are resistant to killer toxins but do not produce it (Bevan and Makower, 1963; Woods and Bevan, 1968; Medina *et al.*, 1997). The killer toxin can change the nitrogen metabolism by decreasing the ion gradient across the membrane of the sensitive yeasts and consequently interrupting the coupled transport of protons and amino acids (De la Peña *et al.*, 1981). The toxin also causes the cellular loss of small metabolites such as ATP, glucose and amino acids (Bussey, 1974). Killer toxins can inhibit wine fermentation by sensitive yeasts (Van Vuuren and Wingfield, 1986; Radler and

Schmitt, 1987; Carrau *et al*., 1988, 1993). The interactions between killer and sensitive yeasts and the effect on nitrogen metabolism in winemaking conditions have been studied extensively (Shimizu, 1993; Medina *et al.*, 1997; Torrea-Goñi and Ancín-Azpilicueta, 2002).

Moulds present on the berries may produce mycotoxins to which *Saccharomyces* is susceptible (Lafon-Lafourcade and Ribéreau-Gayon, 1984; Bisson, 1999). In addition, plant produced compounds (the phytoalexins) and enzymes (the pathogenesis-related proteins) may impact yeast growth (Bisson, 1999) since these compounds are produced in response to fungal infection.

Sulphites

Sulphites are highly toxic to microorganisms. Molecular SO_2 is more active at low pH. Thus molecular SO_2 is extremely active against yeasts in low pH (3-3.5) must. Sulphite toxicity to yeast is largely dependent on the level of SO_2 accumulation in the cell. Once inside the cell, the sulphites cause a rapid decrease in the intracellular ATP level, resulting in cell death (Hinze and Holzer, 1986). Excessive use of SO_2 is toxic to yeast cells (Alexandre and Charpentier, 1998).

Agricultural residues

Fungicides and pesticides used in the vineyard may negatively affect yeast viability if present at high enough residual concentrations at the time of harvest (Lafon-Lafourcare and Ribéreau-Gayon, 1984; Bisson, 1999). These residues can act directly or indirectly to inhibit yeast growth during fermentation (Specht, 2003).

Physical factors

Excessive must clarification

Excessive must clarification can often cause sluggish fermentation due to the loss in fatty acid content, sterol content and macromolecules (Alexandre and Charpentier, 1998). The level of solids also affect alcohol tolerance, therefore the choice of an alcohol tolerant strain is more important in a clarified juice than a high solid must (Henschke, 1997). Must clarification affects the assimilation of nitrogen compounds and reduces nutrients and eliminates fatty acids, especially many unsaturated fats. As a result the amino acid transport system is affected (Ayestarán *et al*., 1995; Ancín *et al.*, 1998; Ayestarán *et al*., 1998).

pH

Saccharomyces is tolerant to low pH fermentations and can grow in a juice pH range of 2.8 to 4.2 (Lafon-Lafourcade and Ribéreau-Gayon, 1984; Heard and Fleet, 1988; Bisson, 1999). The pH also affects the anti-microbial activity of sulphite which could potentially influence yeast viability (as mentioned in *Sulphites* section) and fermentation performance.

Temperature extremes

Temperature extremes during fermentation can severely affect yeast growth and metabolism (Specht, 2003). Ethanol resistance is also influenced by temperature (Heard and Fleet, 1988; Bisson, 1999; Bisson and Butzke, 2000). At higher temperatures, the cell membrane fluidity increases and ethanol can enter the cell more readily, adversely affecting metabolism and cell viability. Cooler temperatures may enhance ethanol resistance by increasing sterol levels in yeast cell membranes (Suutari *et al*., 1990; Torija *et al*., 2003) resulting in lower accumulation of intracellular ethanol (Lucero *et al*., 2000).

Microbial incompatibility

Initial high populations of non-*Saccharomyces* yeast and bacteria increase the risk of stuck and sluggish fermentations to occur (Drysdale and Fleet, 1989; Bisson, 1999; Edwards *et al.*, 1990, 1998). This is due to competition for nutrients and production of toxic substances. Using unsanitized equipment (cellar hygiene) increases the possibility for microbiological factors such as wild killer yeasts and bacteria (spoilage) influencing the fermentation process. The interactions between *O. oeni* and *S. cerevisiae* are also described by Alexandre *et al*. (2004). Lactic acid bacteria have elaborate nutritional requirements (Buckenhüskes, 1993) and competition for these may inhibit or delay yeast activity during the alcoholic fermentation (Huang *et al.*, 1996; Edwards *et al*., 1998). Lonvaud-Funel (1995) suggests that inoculation of must with starter cultures should take place only after the conclusion of the alcoholic fermentation to avoid the increase of wine volatile acidity due to sugar metabolism by *O. oeni*. Incompatible pairings of wine yeast and lactic acid bacteria is also a possibility. Edwards *et al.* (1998) reported on *Lactobacillus kunkeei* frequently causing stuck fermentations, regardless of the yeast strain present.

Metabolic basis of stuck and sluggish fermentation

The metabolic basis of stuck and sluggish fermentation has been fairly well established. The decrease in rate of sugar consumption is correlated with a decrease in sugar uptake capacity. Glucose and fructose consumption are reduced in response to various environmental or cellular stress conditions such as nutrient limitation (macronutrient and micronutrient), low pH, lack of oxygen, lack of adequate agitation, temperature extremes, presence of toxic substances, presence of other microorganisms, imbalance of cations, and poor strain tolerances (particularly to ethanol or acetaldehyde). All of these have been associated with stuck and sluggish fermentations and have an impact on glucose and fructose transporter expression and activity (Alexandre and Charpentier, 1998).

According to literature (Gafner and Schütz, 1996), fructose levels in some stuck wines are found to be 10 times higher than the glucose concentration. Stuck fermentation can therefore be expected for wines with glucose/fructose ratio smaller than 0.1 (Gafner and Schütz, 1996).

Apart from the influence of nutrients, physical and microbial factors on the metabolism of the yeast which could result in decreased rate of fermentation or even complete fermentations arrest, apoptosis have been suggested as an additional mechanism influencing fermentation (Büttner *et al.*, 2006; Ludovico *et al.*, 2001). Apoptosis refers to the programmed cell death of the yeast cell which is also a regulated suicide program crucial for metazoan development (Madeo *et al*., 2004; Büttner *et al*., 2006).

Cellar management: malolactic fermentation

Despite considerable research (reviewed by Wibowo *et al*., 1985; Britz and Tracey, 1990; Nel *et al.*, 2001), the malolactic fermentation (MLF) process remains to be an imperfectly controlled process and at times MLF can be difficult to get started. The occurrence of MLF problems and the possible causes thereof has been studied less extensively than in the case of alcoholic fermentation problems. In the following sections various factors (summarised in Table 3) which could potentially influence the start and successful completion of MLF will be highlighted.

Inoculation considerations

Malolactic fermentation is a biological process of wine deacidification in which the dicarboxylic L-malic acid (malate) is converted to the monocarboxylic L-lactic acid (lactate) and carbon dioxide (Davis *et al*., 1985). This process is normally conducted by lactic acid bacteria (LAB) isolated from wine, including *Oenococcus oeni* (previously *Leuconostoc oenos*, Dicks *et al*., 1995), *Lactobacillus* spp. and *Pediococcus* spp. (Wibowo *et al.*, 1985). *O. oeni* is the preferred starter culture to conduct MLF due to its tolerance to low pH, high ethanol and SO_2 levels and flavour profile produced (Kunkee, 1967; Wibowo *et al.*, 1985; Tracey and Britz, 1987; Van Vuuren and Dicks, 1993). It has been shown that the ability to perform MLF in harsh conditions is closely related to the physiological properties of the *O. oeni* strain inoculated (Nannelli *et al*., 2004).

Although MLF may occur spontaneously, the fermentation management can be simplified with the introduction of *O. oeni* cultures (Krieger, 1993; Nielsen *et al*., 1996). The lag phase associated with spontaneous MLF (wild/uncultured strains) increase the risk of spoilage organisms and production of volatile acidity (as a result of lactic acid bacterial sugar metabolism) due to the low SO_2 levels. Inoculation with a LAB culture avoids these problems by immediately providing the population (more than $2x10^6$ cells/mL) necessary to conduct MLF. Semon *et al*. (2001) suggests that pre-fermentation inoculation results in increased volatile acidity concentrations. However, the success of MLF is not always guaranteed due to changes in fermentation conditions, grape must composition and microbial competition (Krieger, 1993). Compatibility of yeast and LAB should be considered when time of inoculation is considered. Very often, starter culture failures are due to improper preparation and inoculation procedures. In some cases, starter culture failure may be due to antagonistic interactions between yeast and bacteria.

Nutritional requirements

Malolactic fermentation difficulty could be the result of insufficient nutrients important for the development of LAB (Nygaard and Prahl, 1996). Yeast can reduce the nutrients available to LAB considerably and therefore time of inoculation is critically important to avoid competition for nutrients. For this reason, winemakers often add a nutrient when inoculating with MLF starter cultures to assist their development. This addition is especially important if the must and wine initially has low levels of nutrients or if yeast strains with inherently high nutritional requirements were used. The addition of bacterial nutrients ensures a quick onset and completion of MLF and could also prevent delayed and/or stuck MLF.

TABLE 3

Factors in the cellar environment that could influence malolactic fermentation.

Cellar management: Malolactic Fermentation	
Sections	**Subsections**
Inoculation considerations	
Nutritional requirements	
Inhibitory factors	Ethanol content
	pH
	Temperature
	Sulphur dioxide
Microbial interactions	

LAB have elaborate nutritional requirements (Buckenhüskes, 1993) with limited means of synthesizing growth requiring compounds (Fourcassier *et al.*, 1992; Fugelsang, 1996). *Oenococcus oeni* has very specific and at times very fastidious nutritional requirements to support sufficient growth and development of the bacteria. Studies suggest that wine carbohydrates (Melamed, 1962; Ribéreau-Gayon *et al*., 1975; Dittrich *et al.*, 1980) and amino acids (Mayer *et al.* 1973; Temperli and Kuensch, 1976; Beelman and Gallander, 1979) may be utilised by these bacteria during malolactic fermentation and this metabolism as well as that of organic acids (Pilone *et al*. 1966; Kunkee, 1974; Beelman and Gallander, 1979; Lafon-Lafourcade and Ribéreau-Gayon, 1984; Ribéreau-Gayon *et al*., 1975) can lead to changes in the concentration of constituents which affect sensory quality of wines (Davis *et al*., 1986). Inorganic nitrogen [supplied in the form of diammonium phosphate (DAP)] cannot be used by these bacteria (Ribèreau *et al.*, 2000; Loubser, 2005). Vitamins, especially from the B-group, as well as pantothenic acid, are required. In addition, certain trace elements (including magnesium and manganese) also form part of the very specific nutritional requirements of *O. oeni* (Loubser, 2005). Liu (2002) reviewed the current knowledge on the metabolism of LAB (predominantly oenococci) comprehensively. However, the biochemical mechanisms by which LAB grow in wines are still not clearly understood.

Inhibitory factors

The physico-chemical properties that influence LAB growth are well known, mainly: pH, acidity, ethanol and SO_2 concentrations and temperature (Bousbouras and Kunkee, 1971; Ingram and Butke, 1984; Wibowo *et al*., 1985; Davis *et al*., 1988; Wibowo *et al*. 1988; Henschke, 1993). A study by Vaillant *et al.* (1995) investigating the effects of 11 physico-chemical parameters, identified ethanol, pH and SO_2 as having the greatest inhibitory effect on the growth of LAB in wine. Another argument is that inhibitory substances are accumulated in wine and all these factors could have possible synergistic effects on each other, enhancing the inhibitory effect of a specific factor.

Ethanol content

Lactic acid bacteria are sensitive to ethanol and usually struggle above 13.5% exhibiting very slow or non-existent growth. *O. oeni* is a preferred starter culture due to its tolerance to ethanol. The fatty acid composition of the cell membrane of wine LAB can be modified by ethanol. The viability of these bacteria is affected in particular by the saturated/unsaturated fatty acid ratio (Henick-Kling, 1995). It was shown that ethanol (12% v/v) had an inhibitory effect only on cell growth but malolactic activity was not affected (Capucho and San Romão, 1994).

pH

The effect of pH on the growth rate of LAB in wines is well demonstrated in the literature (Bousbouras and Kunkee, 1971; Castino *et al*., 1975; Liu and Gallander, 1983). Davis *et al.* (1986) showed the rate of bacterial growth and malolactic fermentation increased as wine pH was increased from 3.0 to 4.0. The pH of wine has a selective effect upon the species that grow in wine. Usually, *O. oeni* is the only species isolated from wines with a pH below 3.5 (Davis *et al*., 1986). Generally, LAB favour higher pH's and for

most strains, minimal growth occurs at pH 3.0. Under winemaking conditions, pH's above 3.2 are advised.

The lag phase before MLF, in the case of spontaneous MLF, can be prolonged the lower the pH. The species of LAB dominant in the must or wine is determined by the pH (Bousbouras and Kunkee, 1971). At a low pH (3.2 to 3.4) *O. oeni* is the primary LAB species, different strains of which will dominate throughout MLF. At a higher pH (3.5 to 4.0), *Lactobacillus* and *Pediococcus* dominate over *Oenococcus* (Costello *et al*., 1983).

Temperature

The influence of temperature on the growth of LAB and the occurrence of MLF has been thoroughly researched (Van der Westhuizen & Loos, 1981; Wibowo *et al.*, 1985). Research results confirm that MLF occurs much more rapidly at temperatures of 20°C and above than 15°C and below (Loubser, 1999; Du Plessis, 2005). In the absence of SO_2 the optimum temperature range for MLF is 23 to 25°C. Maximum malic acid degradation will occur at 20 to 25°C. However, these temperatures decrease with an increase in SO_2 concentrations resulting in 20°C being more acceptable. Most strains of *O. oeni* grow very slowly or cease to grow below 15°C. Cells may however remain viable at low temperatures.

Sulphur dioxide

Yeast produce SO_2 during alcoholic fermentation and this may inhibit the growth of LAB (Lonvaud-Funel *et al*., 1988; Henick-Klink and Park, 1994). The levels of SO_2 produced by yeast depend on the yeast strain, availability of nutrients and the presence of compounds in the must (e.g. acetaldehyde) which binds SO_2 (Nygaard and Prahl, 1996). Already in 1994 Henick-Kling *et al.* demonstrated the inhibition of malolactic starter cultures by active growing yeasts due to the production of high levels of SO_2 during the early stage of alcoholic fermentation.

Apart from the selective effect of pH on the growth of LAB, the long-term survival of *O. oeni* under practical wine conditions is determined by the addition of SO_2 (Lafon-Lafourcade *et al.*, 1983). According to other studies (Somers and Wescombe, 1982; Lafon-Lafourcade, 1983), a total SO_2 concentration of more than 50 mg/L generally restricts the growth of LAB in wines, especially at the lower pH values when a greater proportion of the SO_2 is in the undissociated, antimicrobial form. It is therefore not recommended to add SO_2 to must after alcoholic fermentation if MLF is desired (Henick-Kling, 1994).

TABLE 4

Different technologies could be potentially used to monitor fermentations. These are summarised in the following sections and subsections.

Technology To Monitor Fermentation Arrest	
Sections	**Subsections**
Microbiological	Enumeration by traditional plating
	PCR related technologies
	Flow cytometry
Chemical analysis	Chromatographic techniques
	Spectroscopy
	Electrochemical sensors
Chemometrics	

Microbial interactions

Yeast (*S. cerevisiae*) may deplete complex nutrients and growth factors required by LAB and may release bioactive metabolites (SO_2, fatty acids and macromolecules) that can stimulate, inhibit or have negligible effect on the metabolism of LAB (Lonvaud-Funel *et al*., 1988; Edwards *et al*., 1990; Capucho and San Romao, 1994; Henick-Kling and Park, 1994; Rosi *et al.*, 1999; Alexandre *et al.*, 2004). Interactions between co-existing yeast (*S. cerevisiae*) and *O. oeni* can cause problems with MLF. Fermentations of must with low levels of nutrients may cause the yeast used during alcoholic fermentation to produce increased levels of SO_2 which may inhibit MLF. In the case of inoculation before the completion of alcoholic fermentation, bacterial inhibition decreases towards the end of fermentation. This could be explained by the death phase of yeast which reduces the SO_2 produced and the availability of nutrients as a result of yeast autolysis (Nygaard and Prahl, 1996).

The presence of bacteriophages (bacterial viruses) can also cause sluggish or stuck MLF (Henick-Kling, 1994) and can be problematic if wooden barrels used for maturation are contaminated (Berthelot, 2000).

The growth of *Pediococcus* spp. are favoured in high pH wines, resulting in volatile acidity or the production of bacteriocins (antimicrobial proteins or peptides) which may inhibit the growth of *O. oeni* (Green *et al.*, 1997; Van Reenen *et al*., 1998).

King and Beelman (1986) suggested that the growth of *O. oeni* during alcoholic fermentation might be retarded by the production of toxic compounds by yeasts other than ethanol and sulphur dioxide. Alcohol, temperature and pH can modify the fatty acid composition of the cell membrane of wine LAB. In particular the saturated/unsaturated fatty acids ratio affects the viability of these bacteria (Henick-Kling, 1995).

TECHNOLOGY TO MONITOR FERMENTATION

Various technologies are currently available to monitor and investigate the progress of fermentation. This includes microbiological techniques to evaluate whether sufficient cell numbers are present for successful alcoholic (Boulton *et al.*, 1996) or malolactic (Semon *et al*., 2001) fermentation or to determine the identity of spoilage organisms present (Delaherche *et al.*, 2004) in the fermentation. Apart from valuable microbiological information, chemical and spectral data also provide important information to successfully manage both alcoholic and malolactic fermentation. The determination of specific marker compounds such as acetic acid which could serve as an indicator of problematic fermentations (Malherbe, 2007). The combination of microbial, chemical and spectral data could be further exploited with the application of various data analysis techniques (chemometrics) to investigate fermentation patterns, trends or even to identify potential stuck or sluggish fermentations (Malherbe, 2007). The following sections and subsections (layout presented in Table 4) will discuss some techniques which could be of value for the investigation of alcoholic and malolactic fermentations.

Microbiological

Enumeration by traditional plating

The identification and enumeration of microorganisms throughout the fermentation process by plating on selective growth media is a standard microbiological technique. However, this method

of enumeration is often time consuming, laborious and could be inaccurate as a result of the possible viable but non-culturable (VBNC) state of microorganisms. Cells in VNBC state are defined by Olivier (1993) as cells which are metabolically active but unable to undergo the cellular division for growth in liquid or on agar. The evolution to a VNBC state is related to the intensity of the stress (Olivier *et al.*, 1995).

Polymerase Chain Reaction (PCR) related technologies

Many molecular techniques have been developed for yeast identification and characterization (Querol and Ramón, 1996; Guillamón *et al.*, 1998; Esteve-Zarzoso *et al.*, 1999; Loureiro and Querol, 1999; Querol *et al*., 2000), the majority of which are culture-dependent techniques.

Real-time or quantitative PCR (QPCR) methods have been developed to enumerate several species of LAB, including those found in wine (Delaherche *et al.*, 2004; Furet *et al.*, 2004; Pinzani *et al.*, 2004; Neeley *et al*., 2005). González *et al*. (2006) reported the use of nested PCR and real-time PCR for the detection (qualitative) and enumeration (quantitative) of acetic acid bacteria in wine conditions.

López *et al*. (2003) demonstrated the use of a PCR-based method to monitor inoculated wine fermentations and ensure the fermentation is conducted by the inoculated yeast. The method is based on the variation in the number and position of introns in the mitochondrial gene *COX1* (López *et al.*, 2003).

One of the most commonly used culture-independent fingerprinting techniques is denaturing gradient gel electrophoresis (DGGE). It is based on the separation of PCR amplicons of the same size but different sequences (Ercolini, 2004). The theoretical aspects of this separation were first described by Fisher and Lerman (1983). Many applications of PCR-DGGE in microbial ecology have been previously described and reviewed (Muyzer *et al.*, 1997; Muyzer and Smalla, 1998; Muyzer, 1999). Applications of PCR-DGGE include the identification of microorganisms, the evaluation of microbial diversity and microbiological quality assessment (Ercolini, 2004). A study by Cocolin *et al.* (2000) demonstrated PCR-DGGE is a viable alternative to standard plating methods for qualitative assessment of the microbial constituents in model wine fermentations. The quantitation of DGGE profiles however, is problematic due to the complex nature of multitemplate PCR (Wagner *et al*., 1994; Suzuki and Giovannoni, 1996; Hansen *et al.*, 1998; Polz and Cavanaugh, 1998).

Flow cytometry

In recent years, flow cytometry (Bruetschy *et al*., 1994; Bouix and Leveau, 2001; Malacrino *et al*., 2001, 2005; Thornton *et al*., 2002; Boyd *et al*., 2003) has been used to monitor live and dead yeast cell concentrations during fermentation (Chaney *et al.*, 2006). This technique allows the counting, examining, and sorting of microscopic particles suspended in a stream of fluid. Validation of this method has been performed by comparison with other viability analysis techniques such as haemocytometry and plating (Fiala *et al*., 1999; Thornton *et al*., 2002). Thiazol orange, a permeant DNA-reactive stain that enters live and dead cells, fluoresces at 530 nm and is used to differentiate cells from debris. Additional staining with propidium iodide, an impermeant DNA-reactive stain which cannot penetrate cells with intact membranes, fluoresces at 625 nm and differentiates live and dead cells.

Since cell viability stains often rely on membrane integrity, they do not necessarily report on the metabolic activity of cells. Varela *et al*. (2004) suggested that fermentation rate is a combination of intracellular (metabolic) activity and the mass of cells actively fermenting. It may therefore be more informative to monitor the metabolic activity or yeast 'vitality' than the cell viability alone. This information could possibly serve as a better predictor of stuck fermentations by determining the physiological state of the yeast populations using flow cytometry and fluorescent viability staining (Bouchez *et al.*, 2004) combined with fluorescent vitality staining.

Chemical analysis

An accurate measurement of various chemical components throughout the winemaking process is a necessity in determining causes of stuck fermentations. As a result of the development and improvement of technology, the focus of wine analysis has shifted towards evaluating and establishing high-throughput analytical methods.

Chromatographic techniques

High Performance Liquid Chromatography (HPLC) is an analytical technique for the separation and determination of organic and inorganic solutes in a variety of samples. Analysis of the major organic acids, carbohydrates, glycerol and ethanol in wine and grape must using HPLC systems with refractive index (RI) and ultraviolet (UV) detection have been reported in numerous studies (McCord *et al.*, 1984; Frayne, 1986; Falque Lopez and Gomeze, 1996; López-Tamames *et al*., 1996; Michnick *et al.*, 1997; Aragon *et al*., 1998; Castellari *et al.*, 2000; Reynolds *et al*., 2001; Palacios *et al*., 2002). The coupling of HPLC and FT-IR for the determination of carbohydrates, alcohols and organic acids was presented by Vonach *et al.* (1998). Since most compounds absorb in the infrared region, FT-IR can provide qualitative information about the compounds and can be regarded a general detector for liquid chromatography (reviewed by Somsen and Visser, 2000). Edelmann *et al.* (2003) reported on another HPLC application with diamond attenuated total reflectance (ATR)-FT-IR detection for the determination of carbohydrates, alcohols and organic acids in red wine.

Gas chromatography (GC) is a technique almost routinely used to determine the volatile composition of wine and fermenting must. Several studies using GC in combination with mass spectrometry (GC-MS) and/or additional sorptive extraction techniques such as solid phase micro extraction (SPME), solid phase dynamic extraction (SPDE) and solid phase extraction (SPE) have been reported (Ferreira *et al*., 1996; Vianna and Ebeler, 2001; Alves *et al.*, 2005; Howard *et al.*, 2005; Liu *et al*., 2005; Câmara *et al*., 2006; Esti and Tamborra, 2006). Mallouchos *et al*. (2002) and Hernández-Orte *et al.* (2002) used SPME GC-MS and GC-FID (flame ionization detection) respectively to investigate the relationship between the amino acid profile and aroma profile of wines. Malherbe (2007) showed with a holistic approach that volatile compounds determined with gas chromatographic techniques could be used to discriminate between problem and control fermentations using multivariate data analysis.

Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy has become a popular technique for wine and grape juice analysis (Clark *et al.*,

2006). Many studies have used NMR (^{13}C and ^{1}H) to determine amino acids, organic acids, sugars, alcohols, glycerol, polyphenols, catechin, epicatechin and gallic acid in wine or phenolic extracts (reviewed by Clark *et al*., 2006) and in grape juice or must (reviewed by Clark *et al.*, 2006). Clark *et al.* (2006) reported on monitoring a commercial fermentation using ^{1}H NMR analysis and the aid of chemometrics for the simultaneous determination of a spectrum organic analytes. The potential of this technique as a tool to monitor commercial wine fermentations is however limited due to the availability and access to high field NMR instrumentation.

Infrared spectroscopy (IR) has been used successfully to monitor large scale fermentations, since various compounds are measured simultaneously from a single sample without prior treatment (Urtubia *et al*., 2004). Infrared spectroscopy is a technique based on the interaction of infrared radiation with the vibrations and rotations of atoms of a molecule. Infrared radiation is passed through a sample and the fraction of incident radiation absorbed at a particular energy results in the absorption spectrum. The vibration frequency of a chemical bond in a molecule is related to the energy at which any peak in an absorption spectrum appears. Since all molecules absorb infrared radiation at different wavelengths, an infrared spectrum therefore contains both qualitative and quantitative information of the sample material (Griffiths and de Haseth, 1986; Andersen *et al.*, 2002).

Initially, routine wine analysis used vibrational spectroscopy in the near-infrared (NIR) region (Baumgarten, 1987). The use of FT-IR technology in the MIR region for wine analysis is due to the need for a more accurate determination of more constituents and properties than the NIR method (Nieuwoudt, 2004; Patz *et al*., 2004). Application of FT-MIR spectroscopy is of special interest due to the presence of sharp and specific absorption bands (Schindler *et al*., 1998). Multivariate prediction models are constructed through a calibration process (Eriksson *et al*., 1999; Esbensen, 2002) for predicting wine constituents from the FT-IR absorbance spectrum (Andersen *et al.*, 2002). Recently, this technique has been extensively evaluated for quantification purposes in industrial applications (Patz *et al*., 1999; Dubernet and Dubernet, 2000; Gishen and Holdstock, 2000; Kupina and Shrikhande, 2003; Nieuwoudt *et al*., 2004).

Naumann and co-workers suggested Fourier transform infrared (FT-IR) spectroscopy as a rapid and inexpensive method to identify microorganisms (Naumann, 1985; Naumann *et al*., 1988, 1990, 1991; Helm *et al*., 1991). Absorption of infrared light by cellular compounds results in a fingerprint-like spectrum that can be identified by comparison to reference spectra. The success of the method is therefore directly dependent on the complexity of the reference spectrum library. The application of FT-IR spectroscopy for the identification of microorganisms was firstly reported in the food industry for some species of the genera *Lactobacillus* (Curk *et al*., 1994), *Actinomyces* (Haag *et al.*, 1996), *Listeria* (Holt *et al.*, 1995), *Streptococcus* (Goodacre *et al*., 1996) and *Clostridium* (Franz, 1994). Additional research reported the identification of yeasts by FT-IR (Serfas *et al*., 1991; Henderson *et al.*, 1996; Kümmerle *et al*., 1998).

FT-IR microspectroscopy is a novel tool to characterize microorganisms (Ngo Thi *et al*., 2000). In this method the spectra of single colonies are recorded by a mid-IR spectrometer coupled to a microscope. Isolation and purification of the organisms to be measured are therefore not necessary. Wenning *et al.* (2002) compared identification by FT-IR macrospectroscopy and FT-IR microspectroscopy and found similar results. Therefore, the time-consuming isolation of organisms prior to identification, as is the case with FT-IR macrospectroscopy, is not necessary.

New technology: Electrochemical sensors

One of the most promising directions for the development of innovative analytical methods is the use of electrochemical techniques. These devices consist of chemical sensor arrays coupled with an appropriate pattern recognition system capable of extracting information from the complex signals. The electronic nose consists of an array of gas sensors with different selectivity, a signal collecting unit and pattern recognition software. It is useful for the analysis of headspace of liquid or solid food samples (Schaller *et al.*, 1998) and numerous attempts using the electronic nose for wine analysis have also been reported (Di Natale *et al*., 1995, 1996). Similar in principal to the electronic nose, the electronic tongue consist of an array of sensors designed for liquids for the evaluation and classification of complex liquids. Various techniques such as conductimetric, potentiometric and voltammetric techniques can be used for the electronic tongue (Winquist *et al*., 2000). Studies using potentiometric electronic tongue for beverage analysis and wine discrimination (Legin *et al.*, 1999, 2003) have been reported. Buratti *et al*. (2004) used an electronic nose and an amperometric electronic tongue to differentiate and classify Italian wines according to region by applying multi-dimensional chemometric techniques. The application of various types of electrochemical sensors (electronic nose and tongue) in combination with other analytical techniques for discrimination and classification in different media has been reported (Toko, 2000; Legin *et al.*, 2004; Ciosek *et al.*, 2005; Cozzolino *et al.*, 2005; Gallardo *et al.*, 2005; Lozano *et al.*, 2005; Ciosek and Wróblewski, 2007; García *et al.*, 2006; Lvova *et al*., 2006; Parra *et al*., 2006). Esti and co-workers reported on the use of electrochemical biosensors for monitoring alcoholic fermentation (Esti *et al*., 2003) and malolactic fermentation (Esti *et al*., 2004) in red wine. The development of electronic tongues and their analytical applications in the food and beverage industry is reviewed by Vlasov *et al*. (2002).

Chemometrics

The application of sophisticated statistical techniques, the so-called "chemometrics", in wine production and laboratory is widely referenced in the literature from several points of view. Multivariate data, such as spectra, are often rich in information. The methods used in chemometrics for the analysis of such large data sets are principal component analysis (PCA Wold *et al.*, 1987; Jackson, 1991) and projections to latent structures (PLS Wold, 1982; Tenenhaus, 1998; Wold and Josefson, 2000; Wold *et al*., 2001). These methods provide a strategy for utilising this richness in information for summarizing data (Wold, 1982; Wold *et al*., 1987), classification and discriminant analysis (Wold, 1976; Stahle and Wold, 1987) and modelling relationships between variables (Wold, 1982; Martens and Naes, 1989; Tenenhaus, 1998; Wold and Josefson, 2000; Wold *et al*., 2001).

Supervised and non-supervised pattern recognition techniques have been used to distinguish different varieties, geographical areas, elaboration processes etc. Câmara *et al.* (2006) used multivariate analysis for the classification and differentiation of

Madeira wines according to the main grape varieties using headspace analysis. Multivariate data analysis have been extensively used to differentiate wines with different geographic origins on the basis of volatile wine compounds (Noble *et al.*, 1980; García-Jares *et al*., 1995; García-Martin *et al*., 1995), specific compounds (hexan-1-ol and cyclo-hexane) (Kwan and Kowalski, 1980), sugars, organic acids and amino acids (Guedes de Pinho, 1994) and trace elements (Day *et al.*, 1995). The value of chemometrics has also been proven a versatile and valuable tool for assessing wine authenticity (Arvanitoyannis *et al*., 1999).

Multivariate regression, such as principal component regression (PCR) and partial least squares regression (PLSR) are standard procedures in chemometrics, which has been used for developing equations for the determination of quantitative parameters in wine and other food industries using the data provided by the spectroscopic techniques.

FT-IR spectroscopy in combination with chemometric data evaluation provides valuable quantitative information even for highly complex problems such as wine analysis. This application provides high-throughput quantitative information which is important for monitoring fermentations.

CONCLUSIONS

Fermentation predictability and wine quality are principally dependent on wine yeast attributes even if a wide range of factors affect the fermentation performances of yeasts. In particular, the ability to adapt to nutritional deficiency and to cope with the presence of inhibitory substances is of vital importance to fermentation performance.

Difficulties arise from a combination of factors and a variety of sources. It is the impact of two or more conditions together that may cause a problem of much greater difficulty than what would have been predicted by a single parameter acting alone. Therefore, each step of the winemaking process needs to be approached with as complete an understanding as possible. The cause of a stuck or sluggish fermentation is rarely the result of one factor in isolation. Generally, various factors would have a synergistic effect on each other, enhancing the effect of a specific factor.

A better understanding of the aspects of wine microorganism physiology will allow us to better match combinations of yeast and bacteria starter cultures with grape varieties, and select the timing of yeast and bacteria inoculations.

Fermentation problems usually arise due to the presence and impact of various stress factors in the yeast and bacteria environment. Some of these stress factors are however unavoidable and others are the result of inappropriate fermentation management decisions. New analytical technologies in combination with chemometrics such as multivariate data analysis could provide powerful tools to monitor industrial fermentations and prevent fermentation problems in the future.

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